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# CEREAL CHEMISTRY

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No. 1

## THE EFFECT OF VARIOUS INGREDIENTS ON THE RATE OF FIRING OF BREAD CRUMB IN THE PRESENCE OF POLYOXYETHYLENE (MONO) STEARATE AND GLYCERYL MONOSTEARATE<sup>1</sup>

E. C. EDELMANN, W. H. CATHCART, and C. B. BERQUIST<sup>2</sup>

### ABSTRACT

Effective methods of retarding the progress of crumb firming during staling of bread include the use of lard as an ingredient, use of maximum water absorption coupled with minimum baking period required to produce bread with moisture content of not more than 38%, obtaining the maximum practical value for the ratio of baking pan volume to dough weight, and use of either polyoxyethylene (mono) stearate or glyceryl monostearate as an ingredient.

Non-fat dry milk solids, sucrose, yeast, and sodium chloride were found to have little effect in delaying the increase in crumb firmness.

The effect of various ingredients and of pan volume to dough weight relationship on increase in crumb firmness was investigated with and without polyoxyethylene (mono) stearate and with and without glyceryl monostearate.

Ever since scientific methods have been applied to the problems of the milling and baking industries, cereal chemists have striven to improve the keeping properties of bread. In the past, many investigators have reported the effect of various ingredients on certain manifestations of staling, such as increase in crumb firmness, reduction in crumb swelling power, reduction in the quantity of soluble starch, and other factors. Most of the work reported on this subject has been reviewed by Geddes and Bice (3).

In recent years, certain materials having surface-active properties and known as emulsifiers have come into use as inhibitors of crumb firming. The effect of various ingredients on increase in crumb firmness in the presence of the two most commonly employed surface-active agents; glyceryl monostearate and polyoxyethylene (mono) stearate, referred to in this paper as poeams, has been studied.

<sup>1</sup> Manuscript received May 19, 1949.

<sup>2</sup> The Great Atlantic & Pacific Tea Co., National Bakery Division, New York 17, N. Y.



### Materials and Methods

The effects on crumb firmness of the following levels of lard, non-fat dry milk solids, sucrose, yeast, sodium chloride, and water were investigated.

|                         |                             |
|-------------------------|-----------------------------|
| Lard                    | None, 3%, and 8%            |
| Non-fat dry milk solids | None, 3%, and 6%            |
| Sucrose                 | 4%, 8%, and 12%             |
| Yeast                   | 1%, 2%, and 3%              |
| Sodium chloride         | None, 2%, and 3%            |
| Water                   | 60%, 64%, 65%, 66%, and 70% |

All percentages of ingredients given in this paper are based on the flour weight as 100%. The effect of these ingredients on increase in crumb firmness and the effect of the relationship between the volume of the baking pan and the quantity of dough baked in it was studied with and without 0.5% poems and with and without 0.5% glyceryl monostearate. Whenever 0.5% glyceryl monostearate was used the absorption was increased 1%.

The bread was made in the laboratory by the sponge and dough process from a hard red winter wheat patent flour. All doughs had an absorption of 65% and contained 3% lard and 3% non-fat dry milk solids unless otherwise noted. Absorption was increased to 68% when 6% non-fat dry milk solids were added and was reduced to 62% when they were omitted. The absorption remained at 65% for all levels of lard, sucrose, yeast, and sodium chloride. Mixing was done with a Hobart Mixer, Model A-200, using a McDuffee type bowl and fork. Sponges had a temperature of 76°F. (24.4°C.) out of the mixer and were fermented for 180 minutes at 80°F. (26.8°C). Doughs had a temperature of 80°F. (26.8°C.) out of the mixer and were divided immediately after mixing. The dough pieces remained at 80°F. (26.8°C.) for 20 minutes between dividing and molding. They were rounded by hand but molded mechanically. Two dough pieces, each weighing 9 ozs., unless otherwise noted, were placed crosswise in the baking pan to form one loaf. The baking pans used measured: inside top 8.5 in. by 4.5 in., inside bottom 7.5 in. by 3.5 in., depth 2.7 in., and had a volume of 87.9 cu. in. All doughs were proofed to the constant height of 9.1 cm. before baking. Baking was done in an electrically heated oven equipped with top and bottom heating elements and a rotating metal hearth. All bread was baked for 25 minutes at 450°F. (232°C.) unless otherwise noted. Each experiment, relating to a particular ingredient or factor being investigated, was done in duplicate, at least, and several were done in triplicate.

Changes in crumb firmness occurring during a period of 96 hours after baking were followed by organoleptic and compressimetric methods. All organoleptic tests were made by the same three labora-

tory workers. The softness of bread was determined by squeezing the loaf by hand, feeling the texture of the crumb, eating the bread, and mashing with the fist the cut surface of the loaf and noting its resiliency. Mechanical tests of crumb firmness were made with the Bloom

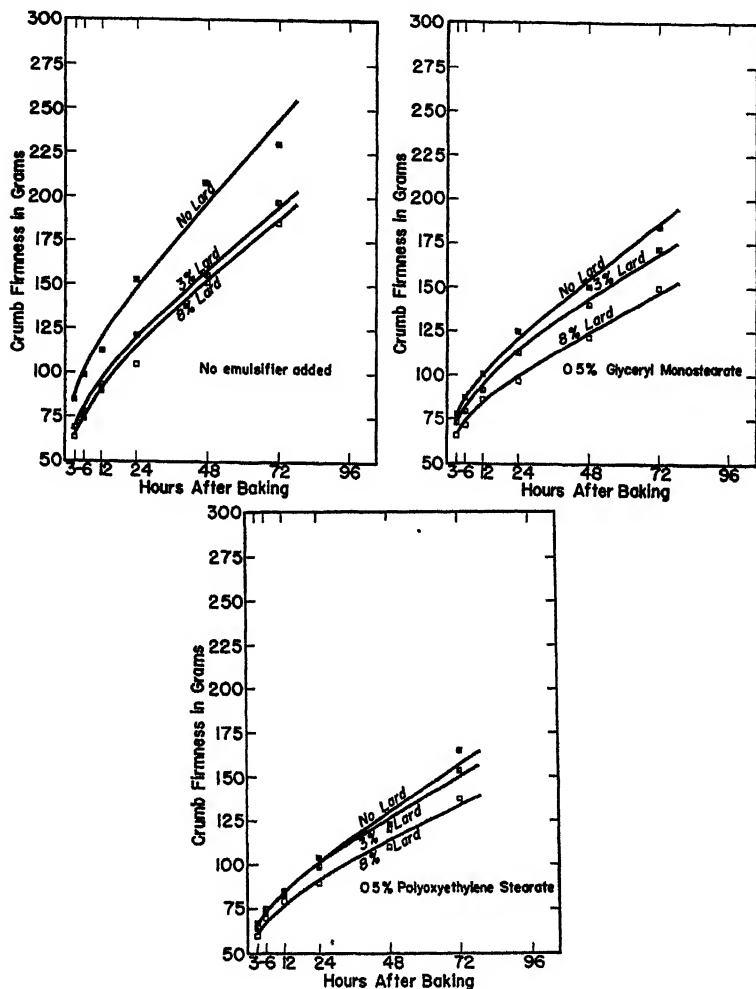


FIG. 1. Effect on increase in crumb firmness of various lard levels; without an emulsifier, with glycerol monostearate, and with polyoxyethylene (mono) stearate.

Gelometer equipped with a plunger of 1 in. diameter as described by Carlin, Hopper, and Thomas (2). In this work a constant strain of 4 mm. was used. Loaves were sliced in a commercial slicer 90 minutes after baking. Excluding both end slices, 17 slices each approximately 0.5 in. thick were obtained from a loaf. Two slices, placed together

on the platform of the Bloom Gelometer, were used for each compressibility measurement. Seven measurements were made on each loaf at each test period and average values were plotted to illustrate the changes in crumb firmness with time. Initial compressibility tests

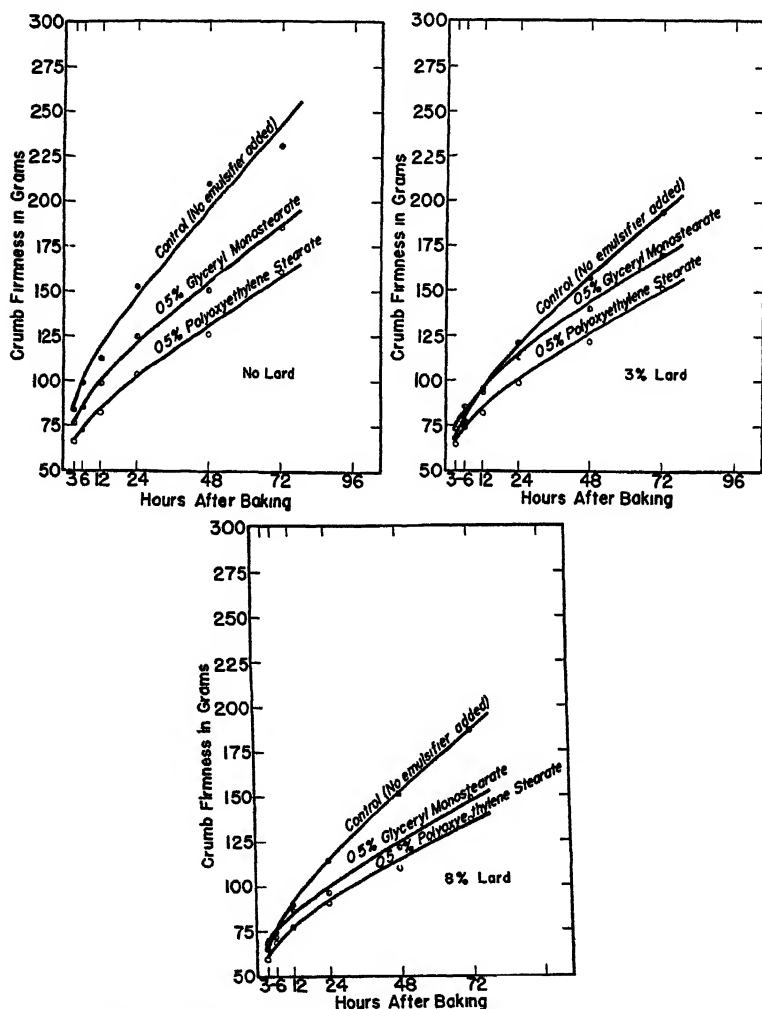


FIG. 2. Effect on increase in crumb firmness of glycerol monostearate and of polyoxyethylene (mono) stearate at various lard levels.

were made as soon as possible; usually within three hours after baking. Further compressibility measurements were made on the same loaves at approximately 24, 48, 72, and 96 hours after baking. All loaves were stored at 80°F. (26.8°C.) between compressibility measurements.

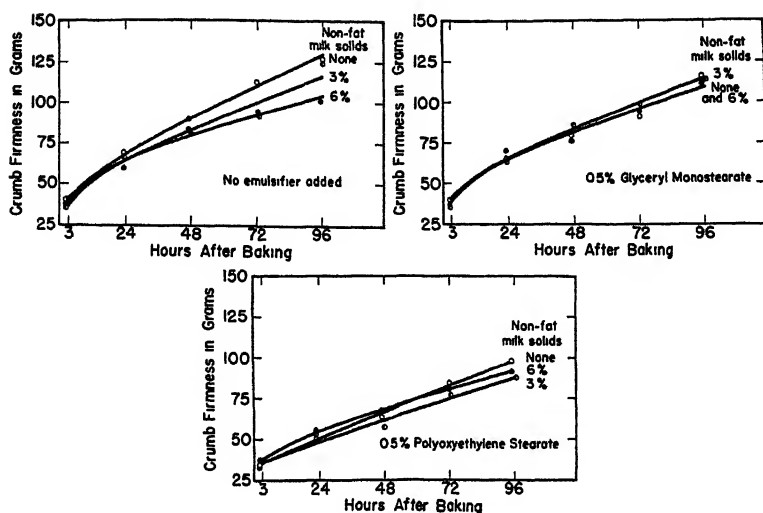


FIG. 3. Effect on increase in crumb firmness of various levels of non-fat dry milk solids, without an emulsifier, with glycerol monostearate, and with polyoxyethylene (mono) stearate.

The determination of increase in crumb firmness was used exclusively in this work as the experimental method because the authors have found that the rate of increase in crumb firming shown by such instruments as the Baker Compressimeter and Bloom Gelometer reflect the findings of the consumer when choosing bread.

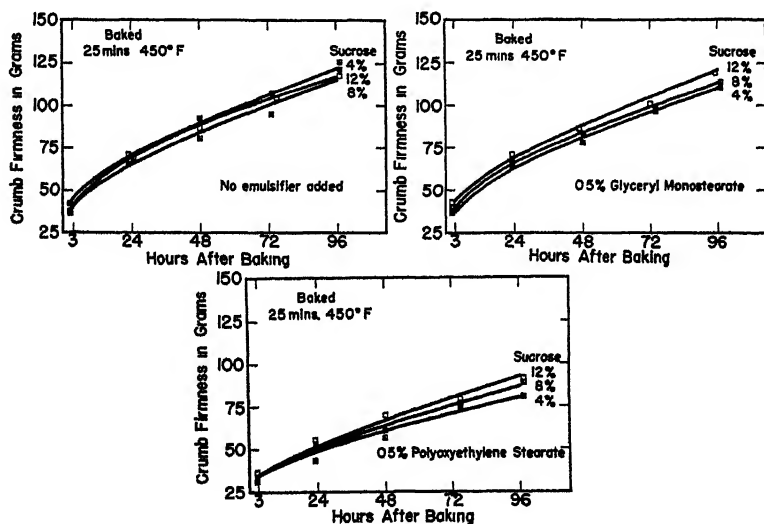


FIG. 4. Effect of various sucrose levels on increase in crumb firmness of bread baked 25 min. at 450°F. (232°C.); without an emulsifier, with glycerol monostearate, and with polyoxyethylene (mono) stearate.

In this paper the term "control" is not limited to one particular formula but describes, in each series, the loaves made without an emulsifier.

### Results and Discussion

*Lard.* Fig. 1 illustrates the effectiveness of lard in slowing the advance of crumb firmness. The addition of 3% lard had the greatest improving effect in the bread made without an emulsifier. Increasing

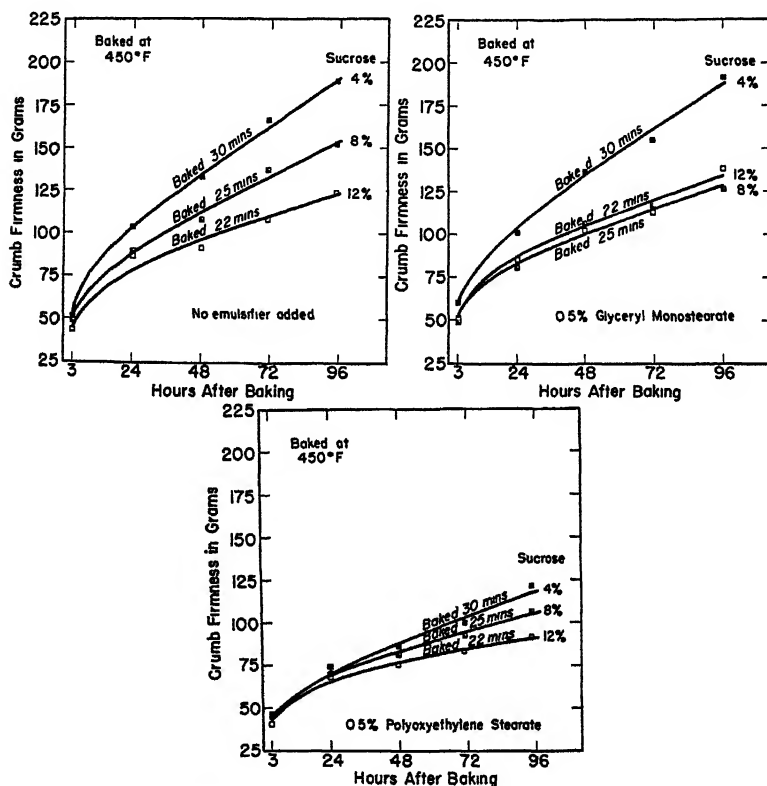


FIG. 5. Effect on increase in crumb firmness of baking at 450°F. (232°C.) bread, made with different levels of sucrose, to the same crust color; without an emulsifier, with glyceryl monostearate, and with polyoxyethylene (mono) stearate.

the lard to 8% showed only slight improvement in the bread made without an emulsifier while bread containing poems and that containing glyceryl monostearate showed a relatively greater improvement from the second lard addition.

Fig. 2 presents the same information in a different manner and illustrates the effectiveness of poems and glyceryl monostearate at different lard levels. Poems and glyceryl monostearate are more

effective crumb firmness retardants in bread made without lard than in bread made with lard. Organoleptic tests indicated that the effectiveness of lard in retarding increase in crumb firming is more readily observed in bread made without an emulsifier.

*Non-Fat Dry Milk Solids.* Fig. 3 shows that increments of 3% non-fat dry milk solids have a tendency to retard the increase in crumb firmness of bread made without an emulsifier. Such improve-

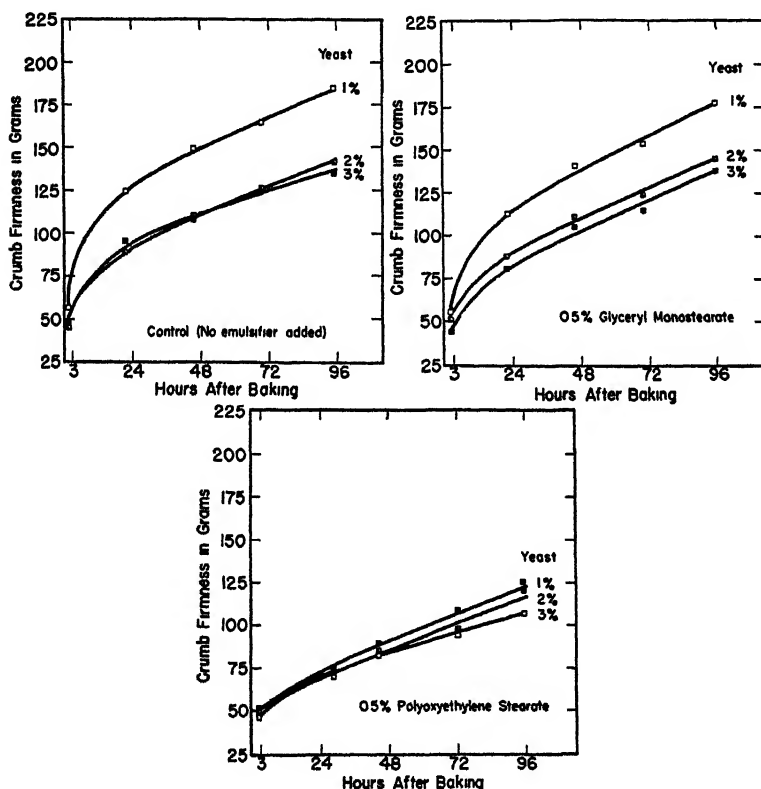


FIG. 6. Effect on increase in crumb firmness of various yeast levels, without an emulsifier, with glyceryl monostearate, and with polyoxyethylene (mono) stearate.

ments are not shown in bread containing either poems or glyceryl monostearate. Although organoleptic tests show the same trend, differences are small and there is little indication that non-fat dry milk solids affect the increase in crumb firmness of staling bread to an important degree.

*Sucrose.* Fig. 4 shows that variations in sucrose content of from 4 to 12% have little effect on the degree or rate of change of crumb

firmness. In bread containing poems and glyceryl monostearate there is a slight tendency toward increased crumb firmness as the sucrose level is increased from 4 to 12%. Organoleptic tests indicated no differences in crumb firmness at various sucrose levels in bread containing poems but did indicate increased crumb firmness at 8 and 12% sucrose levels in bread made with glyceryl monostearate and in bread made without an emulsifier. Because of differences in crust color another experiment was made employing the same sucrose levels as before, but the loaves made with 4 and 12% sucrose were baked until

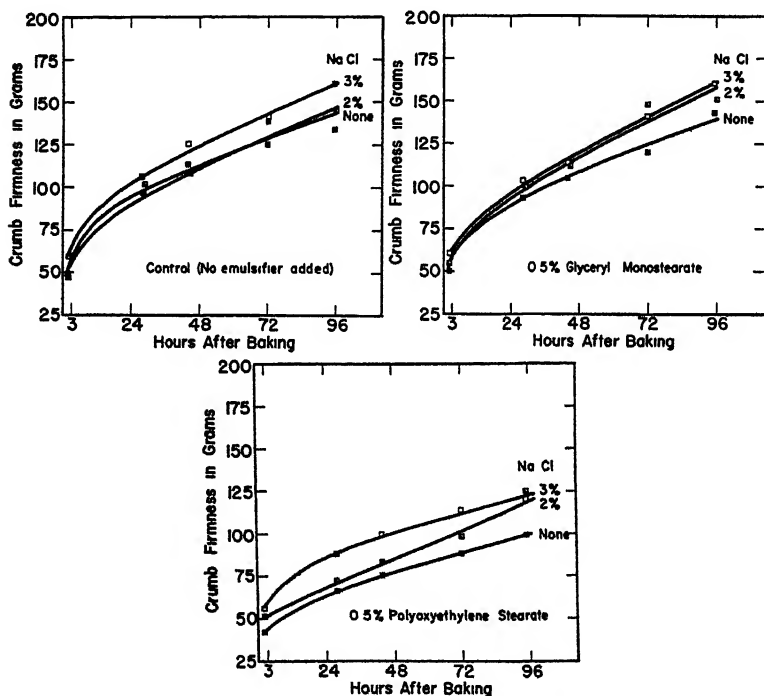


FIG. 7. Effect on increase in crumb firmness of various levels of sodium chloride; without an emulsifier, with glyceryl monostearate, and with polyoxyethylene (mono) stearate.

the crust colors matched visually that of the loaf made with 8% sucrose and baked for 25 mins. Loaves containing 4% sucrose required 30 mins. and those containing 12% sucrose required 22 mins. baking time; all at 450°F. (232°C.). Results are illustrated in Fig. 5. In the second experiment there was a difference in the degree of increase of crumb firmness between loaves having different sucrose levels, especially in the control and glyceryl monostearate loaves. These results were confirmed by organoleptic tests. However, it is likely

that differences in baking time, not sucrose content, accounted for differences in the rate of increase in crumb firmness. The moisture content, as determined by the A.A.C.C. Method (1), of loaves baked for 22 mins. was below 38%.

*Yeast.* Fig. 6 shows that the use of 2% and 3% of yeast tends to produce softer bread than the use of 1% yeast. This effect was barely apparent in bread containing poems. Organoleptic tests confirmed

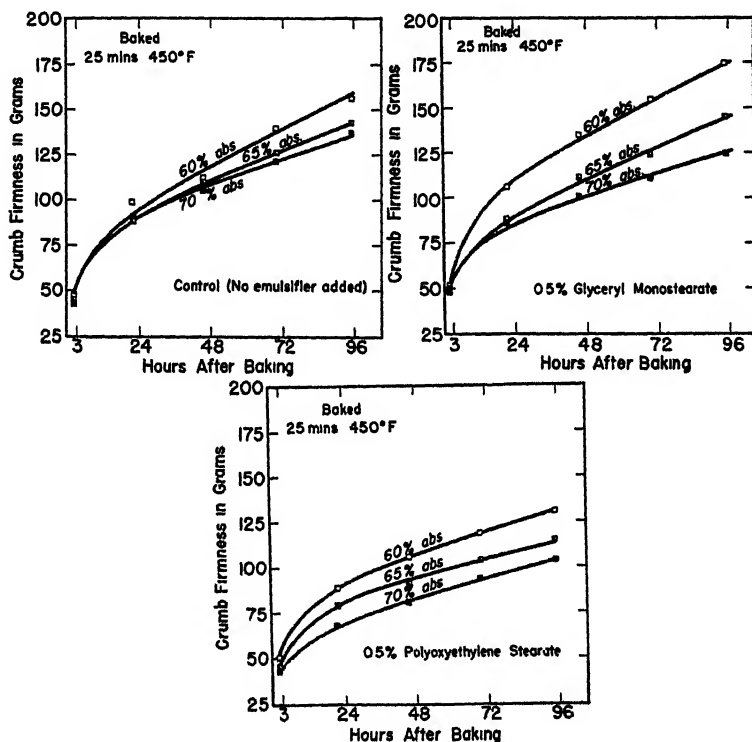


FIG. 8. Effect of various absorptions on increase in crumb firmness of bread baked 25 min at 450°F. (232°C.), without an emulsifier, with glyceryl monostearate and with polyoxyethylene (mono) stearate

these findings. Like all other doughs in this study, these were proofed to a constant height of 9.1 cm. Differences in increase of crumb firmness between loaves made with various quantities of yeast cannot be accounted for by differences in loaf volumes.

| Yeast | No emulsifier added | 0.5% poems | 0.5% glyceryl mono-stearate |
|-------|---------------------|------------|-----------------------------|
| 1%    | 2375 cc.            | 2475 cc.   | 2300 cc.                    |
| 2%    | 2400 cc.            | 2450 cc.   | 2350 cc.                    |
| 3%    | 2350 cc.            | 2575 cc.   | 2375 cc.                    |



Because loaves containing 3% yeast had a strong odor 48 hours after baking, further investigation at higher yeast levels was not made.

**Sodium Chloride.** Fig. 7 shows that in all cases the crumb of bread made without sodium chloride was less firm than that containing 3% sodium chloride. However, this observation probably is without practical value and differences in increase in crumb firmness between loaves made with 2% and 3% sodium chloride were small. Organo-

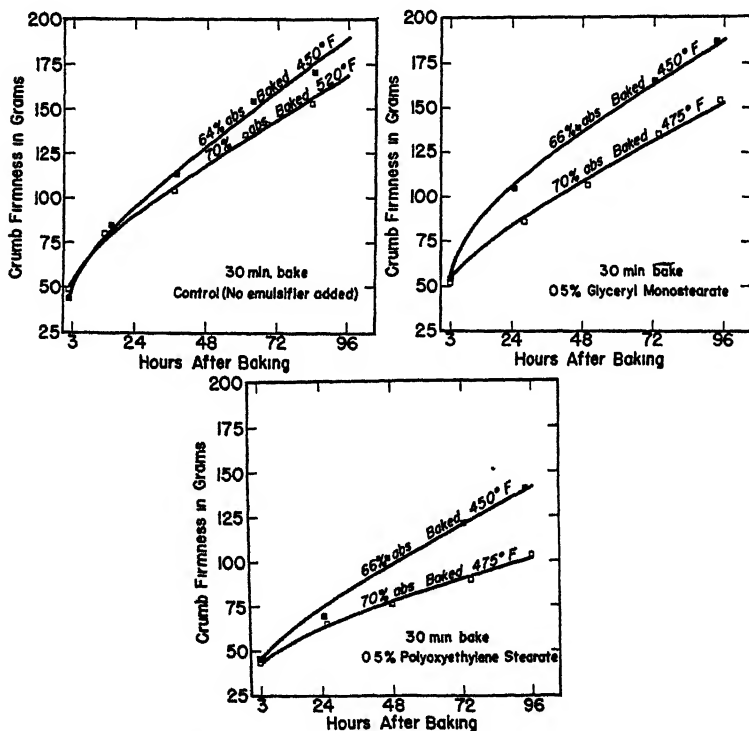


FIG 9. Effect on increase in crumb firmness of bread baked for same period but at different temperatures and absorptions, without an emulsifier, with glycerol monostearate and with polyoxyethylene (mono) stearate

leptic tests did not reflect differences between loaves made with the different levels of sodium chloride and it was noted that, in all cases, the loaves made without sodium chloride were approximately 12% greater in volume than those containing 3% sodium chloride.

**Water.** Two series of breads with absorptions ranging from 60% to 70% were made. Results from both series are illustrated in Figs. 8, 9, and 10. One series with absorptions of 60%, 65%, and 70% was baked for 25 mins. at 450°F. (232°C.). Fig. 8 shows that increased

absorption is effective in retarding the increase in crumb firmness especially in the presence of glyceryl monostearate or poems. Organoleptic tests confirmed this except in mastication where increased absorption brought no observable changes. A second absorption series (illustrated in Figs. 9 and 10) was made using absorptions of 64%

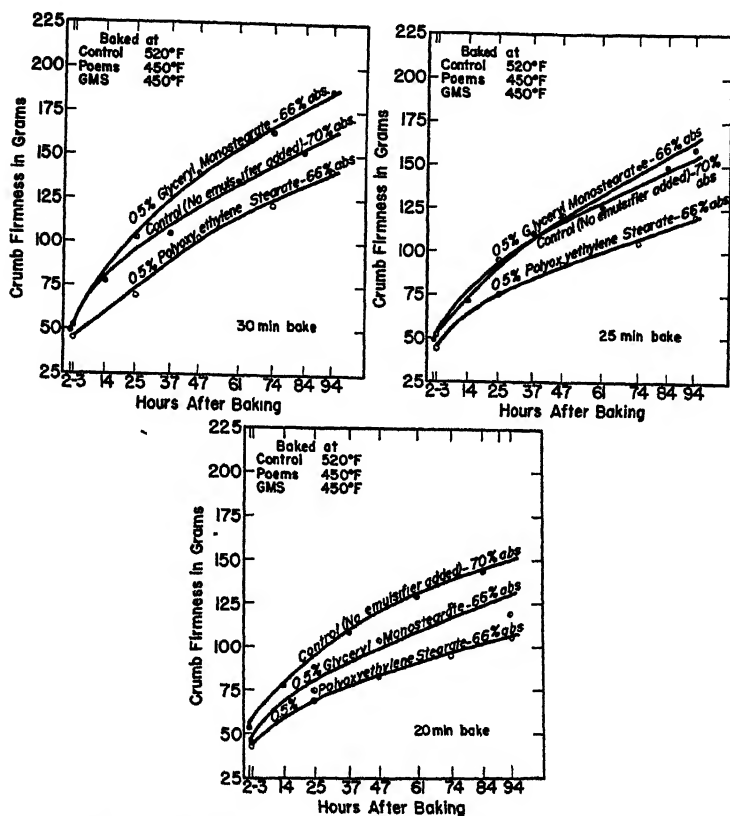


FIG. 10. Effect on increase in crumb firmness of bread baked for different periods, at different temperatures and absorptions, without an emulsifier, with glyceryl monostearate, and with polyoxyethylene (mono) stearate.

and 70% for the control loaves and 66% and 70% for the poems and glyceryl monostearate loaves. Breads with absorptions of 64% and 66% were baked at 450°F. (232°C.); control bread with 70% absorption was baked at 520°F. (271°C.); and those containing poems and glyceryl monostearate and having an absorption of 70% were baked at 475°F. (246°C.). The doughs made with different levels of absorption were baked for three baking times, namely, 20, 25, and 30 minutes. Fig. 9 shows that the effectiveness of increased absorption in retarding the

increase in crumb firmness with time is maintained even when the bread is baked at a higher temperature. Although control bread baked at 475°F. (246°C.) was not available, that baked at as high a temperature as 520°F. (271°C.) was softer than glyceryl monostearate bread with an absorption of 66% and baked at 450°F. (232°C.). Fig. 10 shows that the crumb of control bread, with an absorption of 70% and

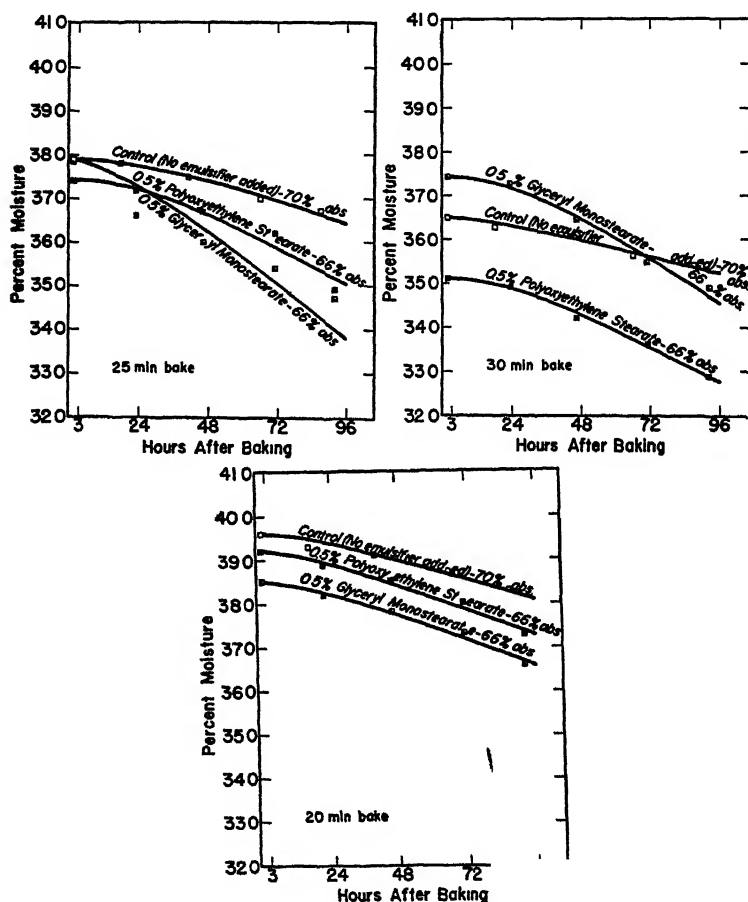


FIG. 11. Moisture content of bread illustrated in Fig. 10.

baked for 25 and 30 minutes at 520°F. (271°C.) is softer at the end of 96 hours than bread containing 0.5% glyceryl monostearate, baked for the same periods at 450°F. (232°C.), and having an absorption of 66%. This is not so when these same breads are baked for 20 mins. However, Fig. 11 shows that all breads baked for 20 mins. have a

moisture content (1) above 38% while those baked for 25 and 30 minutes are below 38%.

*Relationship of Pan Volume to Dough Weight.* It was of interest to investigate the effect of variations in the ratio of pan volume to dough weight on the rate of change in crumb firmness. As in the ingredient experiments, doughs were made without an emulsifier, with 0.5% poems, and with 0.5% glyceryl monostearate. Two fermented

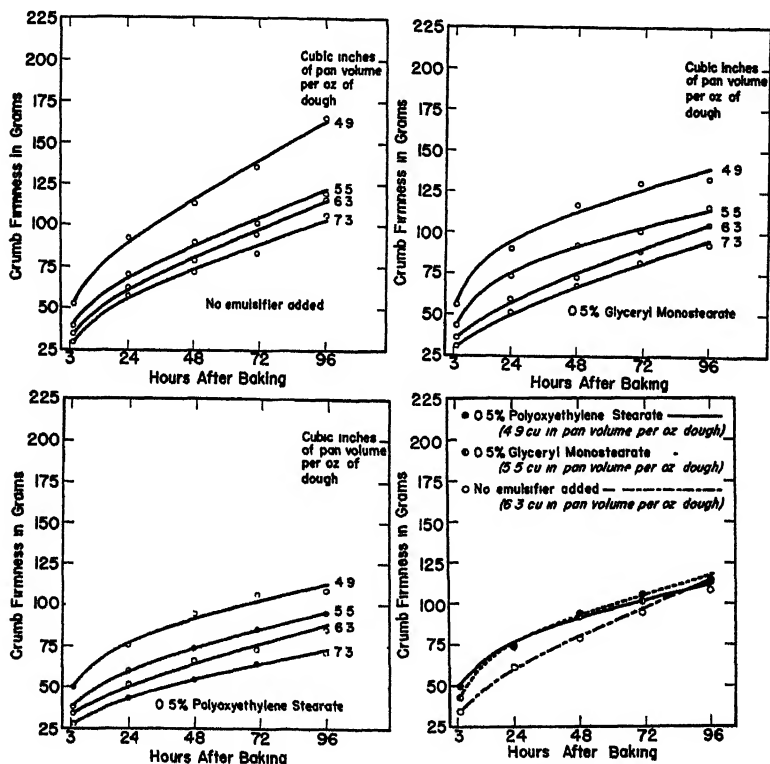


FIG. 12 Effect on increase in crumb firmness of relationship between pan volume and dough weight, without an emulsifier with glyceryl monostearate, and with polyoxyethylene (mono) stearate. Also the effect of various pan volume—dough weight factors, singly and in conjunction with glyceryl monostearate or polyoxyethylene (mono) stearate.

dough pieces, each pair having a total weight of 18, 16, 14, and 12 ozs., were baked in pans having a volume of 87.9 cubic inches giving 4.9, 5.5, 6.3, and 7.3 cubic inches of pan volume per oz. of dough, respectively. Fig. 12 shows that the rate of increase in crumb firmness, over the entire range, was decreased as the number of cubic inches of pan volume per ounce of dough was increased. Fig. 12 also shows that bread made without an emulsifier and having 6.3 cu. in. of pan volume

per oz. of dough was as soft during a 96 hour period as bread containing 0.5% poems and having 4.9 cubic inches of pan volume per oz. of dough; and also as soft as bread containing 0.5% glyceryl monostearate and having 5.5 cubic inches of pan volume per oz. of dough.

The results of this study indicate factors effective in delaying the advance of crumb firmness are: the use of either poems or glyceryl monostearate; the use of lard as an ingredient; the maximum absorption coupled with the minimum baking period required to yield bread having a moisture content, one hour after baking, of not more than 38%; and obtaining the maximum value for the ratio of baking pan volume of dough weight, consistent with the maintenance of bread quality. Non-fat dry milk solids, sucrose, yeast and sodium chloride were found to have little effect in delaying the increase in crumb firming.

#### Acknowledgment

The authors express their thanks to R. G. Schmidt and J. G. Braunreuther, baking technicians, who performed the baking tests on which this study is based.

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# A STUDY OF THE EFFECTS OF VARIOUS TECHNIQUES ON THE MEASUREMENT OF THE FIRMNESS OF BREAD BY THE BAKER COMPRESSIMETER <sup>1</sup>

L. B. CROSSLAND and H. H. FAVOR <sup>2</sup>

## ABSTRACT

The Baker Compressimeter was used to study the force-deformation relations in bread crumb. Large variations in thicknesses of prisms of bread crumb do not alter these relations when the dimensions of the plunger equal or exceed those of the prism. Whole slices were found to be undesirable as test pieces because of the influence of shear at the perimeter of the plunger. The slope of the softness curves produced by fixed force techniques cannot be used alone to indicate staling rate because of anomalous results. The slope of firmness curves, as indicated by fixed deformation techniques, is fairly constant, as bread ages beyond 12 hours; so tests at 18 and 66 hours are indicative of the firming trend. Proper sampling in fixed deformation techniques can eliminate the influence of random differences in firmness of loaves on rates of firming, but these influences cannot be avoided by sampling in fixed force studies. Fixed deformation technique was found to have definite advantages over fixed force technique. It was found that the results of the two techniques could not be compared by the simple use of reciprocals.

Though the mechanism or mechanisms of bread staling are not fully understood, it is recognized that many aspects of staling are due to alterations in the starch and that certain other changes in bread not known to be attributable to the starch do, however, parallel the changes of the starch. The most pronounced and widely recognized change is that which results in firming of bread. Several objective tests have been proposed for evaluating the degree of staleness of bread. Those involving the measurement of changes in the force-deformation relationships of the crumb have proven to be most useful, because they correlate so well with consumer evaluations.

With few exceptions, these force-deformation studies have been made using a machine of the type described by Platt (4) or the Baker Compressimeter (5) which is essentially only an improvement on the Platt machine. No two investigators have used the same test conditions. Some operators have tested whole slices of bread, others, only sections of crumb. Some have recorded values for force for a fixed deformation; others have reported deformation for a fixed force. There has been considerable variation in the maximum values of force and deformation in such studies.

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Geddes and Bice (3) completely reviewed the literature on bread staling and recommended that methods of evaluating staleness be standardized, so that results of various investigators might be compared.

The object of this study was to determine the effects of various fixed conditions on the values obtained by the compressimeter method for determining the staleness of bread, and to establish an optimum set of conditions and procedures which will reflect the changes that occur in bread as it ages.

### Experimental

The Baker Compressimeter equipped with a plunger 32 mm. square attached at the position nearest the fulcrum was used in all test work, except as noted.

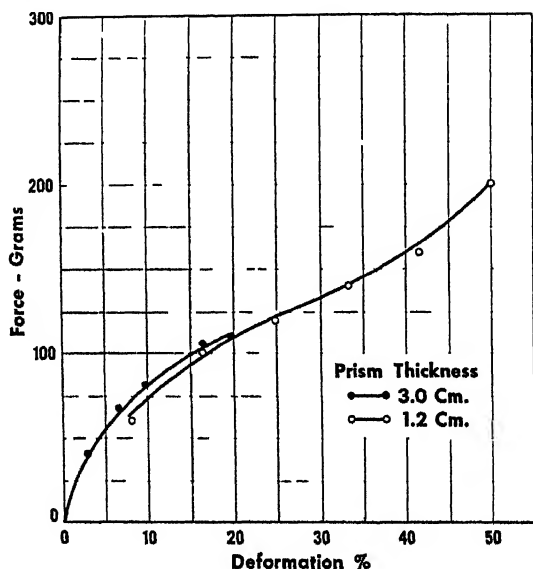


Fig 1. Effect of prism thickness on the profile of the force-deformation curve of bread crumb.

The bread used in these tests was made from an average commercial formula by the sponge-dough process explained by Favor and Johnston (2) and was stored at 86°F. during the test period, except as noted.

*Effect of Thickness of Test Prism.* Rectangular prisms of 18-hour-old bread 32 mm. square, both 12 mm. and 30 mm. thick, were tested in the Baker Compressimeter by taking readings of force at 1 mm. intervals, as the drum rotated.

• When force was plotted against per cent deformation (Fig. 1), it was found that overlapping portions of the curves were quite similar, indicating that large variations in slice thickness did not alter the

nature of force-deformation curves. Platt & Powers (5) noted the advantages of using slices of commercial thickness. Inasmuch as thickness of the test prism is here indicated to have little qualitative effect on results, a thickness of 12 mm. was used in subsequent tests. A prism of these dimensions (32 mm. square by 12 mm. thick) is referred to as a standard prism.

*Effect of Age on Stress-Strain Relations in Bread Crumb.* Force-deformation curves of bread at 4, 18, 42, 66 and 90 hours of age were obtained by plotting forces required to compress a standard prism 1 to 6 mm.

It will be noted in Fig. 2 that the profile of the curve changes very little with age, though the crumb is indicated to be progressively

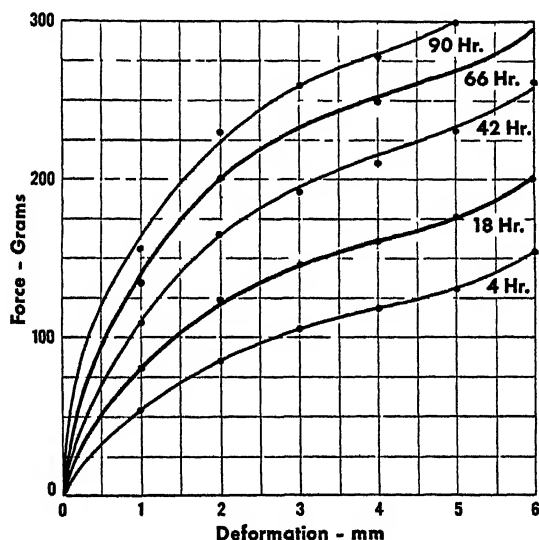


Fig 2. The influence of aging on the profile of the force-deformation curve for bread crumb

firmer at any deformation. It is interesting that in this family of curves the slopes are a minimum between 3 and 4 mm. deformation in each case (Table 1). It is likely that the high force required to produce the 1st mm. deformation is due to the arch type cell structure. As the cells become deformed, the force required for additional deformation decreases, until the bread loses its characteristic texture and approaches a continuous mass.

Obviously, in a practical test, it is not possible to make tests every few hours. The changes occurring in bread during the period from 12 to 72 hours are most interesting to the consumer since this is the period during which most bread is eaten. However, 12 and 72 hour tests are



TABLE I  
SLOPE OF FORCE-DEFORMATION CURVES OF BREAD AT VARIOUS AGES

| Force at .....<br>Minus force at..... | 1<br>0 | 2<br>1 | 3<br>2 | 4<br>3 | 5<br>4 | 6 mm.<br>5 mm. |
|---------------------------------------|--------|--------|--------|--------|--------|----------------|
| Age in hours                          |        |        |        |        |        |                |
| 4                                     | 54*    | 31     | 21     | 13     | 13     | 22             |
| 18                                    | 81     | 42     | 23     | 15     | 15     | 24             |
| 42                                    | 109    | 56     | 27     | 17     | 22     | 31             |
| 66                                    | 134    | 67     | 28     | 19     | 21     | 27             |
| 90                                    | 156    | 74     | 29     | 19     | 22     | —              |

\* Grams per differential millimeter compression (Fig. 2).

highly inconvenient when the bread is laboratory baked. The 12 hour test is due after normal working hours, and the 72 hour test is due at a conflicting hour, where a daily baking schedule is in effect. Tests at 18 and 66 hours cause no conflicts and give results indicative of the firming trend.

*Slice vs. Prism.* Using standard prisms and slices 12 mm. thick, force was observed at 1 mm. intervals up to 6 mm. on bread 18 and 66 hours old.

The results in Fig. 3 show that the profiles of the curves for prisms and whole slices are somewhat dissimilar.

When the dimensions of the prism are equal to or less than those of the plunger, strain is entirely compressive. However, in the whole

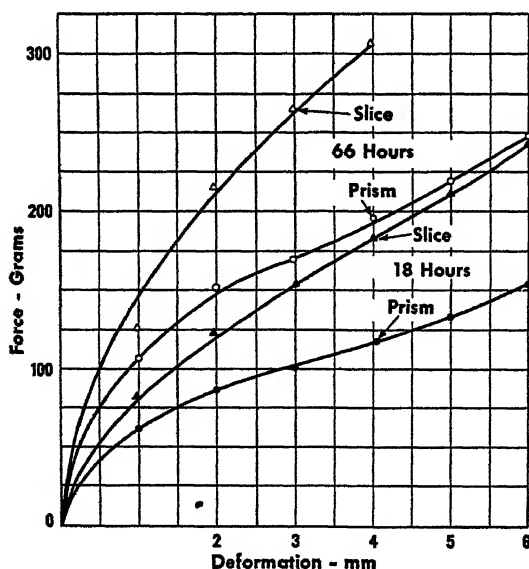


Fig. 3. Contrasting profiles of force-deformation curves for slices and prisms of bread crumb.

slice there is resistance offered to the plunger by the crumb at the perimeter which is in shear. The plunger produces a cup shaped depression in a whole slice, and when the plunger is released, after a high deformation, the outline of the plunger remains well defined indicating more pressure exerted at the perimeter than under the face of the plunger.

In tests of prisms and whole slices of a given type of bread, both 12 mm. thick, a fixed relation existed between forces required for a given deformation of a slice and prism. This relation was relatively constant as the bread aged, tending to confirm the observation of others that testing of whole slices under a given plunger is equivalent to increasing prism and plunger area; however, this ratio of forces was a function of the extent of deformation (Table II).

Forces required to compress a prism of crumb and a slice comparable amounts are obviously related in a complex manner which involves plunger shape, and area, extent of deformation, and, probably,

TABLE II  
RELATIVE EFFECTIVE PLUNGER AREAS IN COMPRESSION TESTING  
OF SLICES OF BREAD

| Deformation in mm | 1st Day | 3rd Day |
|-------------------|---------|---------|
| 1                 | 1.34*   | 1.27    |
| 2                 | 1.43    | 1.42    |
| 3                 | 1.45    | 1.55    |
| 4                 | 1.55    | 1.55    |
| 5                 | 1.59    | —       |

\* Calculated as gram<sup>4</sup> force required for indicated deformation of a whole slice divided by grams force required to compress a prism the same area as the plunger the same amount.

tensile strength of crumb. It appears inadvisable to measure values influenced by more than one characteristic of bread, when comparison with results of other investigators is contemplated.

*Fixed Deformation vs. Fixed Force.* The data in force-deformation studies have been presented graphically in two different manners. One of the most widely used means of presenting the data has been as compressibility or so-called softness curves, which are of hyperbolic type, so there is no curve parameter which may be used to illustrate staling rate. Recently, the trend among investigators has been to plot data as firmness against time; this produces a line with a characteristic slope.

As bread ages beyond the first 12 hours of rapid change, firmness is nearly a straight line function of time. Only two points are required to plot such a curve, whereas a large number are required to plot a curve of hyperbolic type. Because the differences in firmness between loaves of the same batch of bread are often greater than the differences

in firmness of the two halves of a loaf, it is preferable to test half of each loaf under observation at each time interval. In this way, the influence of random differences in firmness of loaves is minimized, and differences in firmness at different test times are attributable to aging. To obtain points required to plot the hyperbolic type curves, different loaves must be used at each time interval because the loaves cannot be symmetrically divided into more than two portions for compression tests. Readings at different times, therefore, are influenced by random differences in firmness of loaves, as well as by aging of the bread.

Fixed force studies were made by reading deformation at fixed force values while the machine was in operation, in contrast to taking

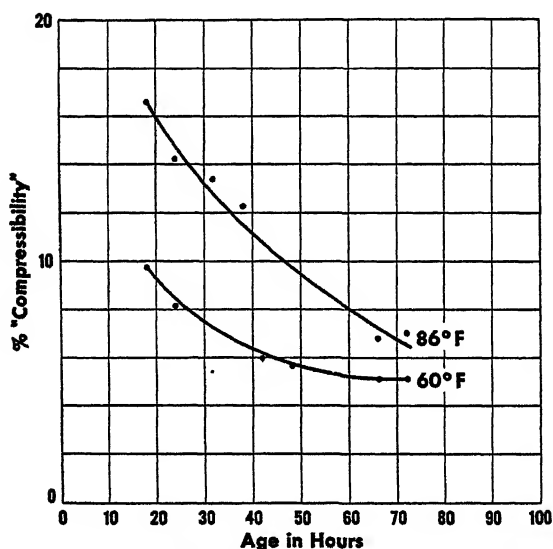


Fig. 4. The influence of storage temperature on "slope of the compressibility curve" for bread crumb under a fixed stress of 11.7 gms/cm<sup>2</sup>.

equilibrium values. The nature of the curves was similar, although higher forces were required for a given deformation when force was read with the machine running.

Bread stored at 86°F. was tested and the values plotted as "compressibility" as is customary in fixed force studies. As indicated in Fig. 4 the curve is flatter for bread stored at 60°F. than for that stored at 86°F. The "slope" of such curves has been used alone as an index of staling rate. From these curves, then, it would appear that bread stored at 60°F. stales less rapidly than bread stored at 86°F., a conclusion which is in conflict with the observations of all who have studied the influence of temperature on bread staling. If the fact that soft-

nesses at the earliest test are not equal is considered, the evaluation becomes complicated and the manner in which allowance should be made is unknown.

*Fixed Strain.* Standard prisms tested at 4 to 90 hours at fixed deformations give curves shown in Fig. 5. Though the firmness values vary as the deformation, the slopes during the period from 12 to 90 hours are roughly equal for deformations from 2 to 4 mm. It would appear, therefore, that the slope of firmness-age curves is a parameter characteristic of the material tested and is not affected by moderate

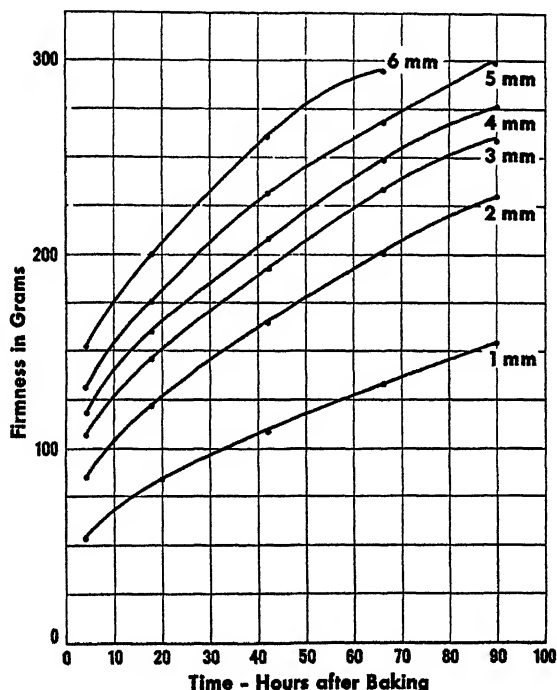


Fig. 5. The influence of deformation on the time-firmness relations in bread crumb.

changes in technique with respect to per cent deformation, provided that per cent deformation is fixed for the duration of the test.

Additional techniques suggested in *Cereal Laboratory Methods*, such as the one where the force on the prism is maintained at constant value and the deformation at definite time intervals is observed (1), have been briefly studied and found to be time consuming and of little, if any, merit, chiefly because curves rather than point values are obtained at each test time. The curves can be interpreted only with difficulty, if at all.

Of the techniques studied, the fixed deformation procedure was judged to have advantages far greater than any noted for fixed force technique.

Attempts have been made by some investigators to compare data obtained by fixed deformation studies with those obtained under fixed force conditions. This has been done by plotting both as firmness or as "compressibility." To do this, the reciprocal values of the appropriate set of data are obtained. If, under the conditions of both tests, force were proportional to deformation in the control breads, at all respective test times, it would be possible to make comparisons by use of reciprocals. If, however, for either or both tests force is not proportional to deformation up to maximum deformation at each test

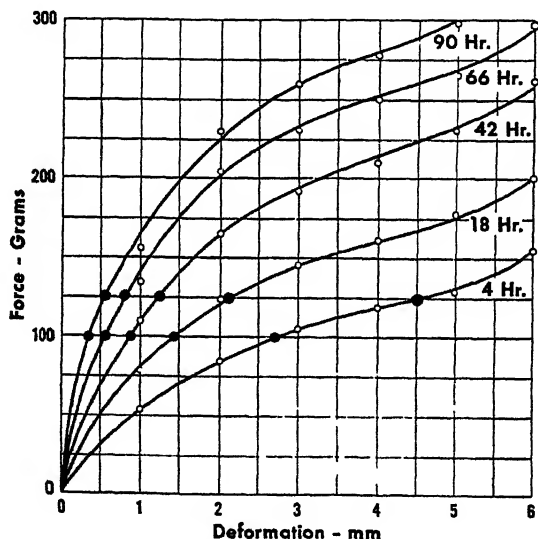


Fig. 6. The dense circles at ordinates of 100 grams and 125 grams indicate the data used in producing the broken curves of Fig. 7. The ordinates at 1 mm. indicate the data used to produce the solid curve of Fig. 7.

time, the use of reciprocals is not justified, so such comparisons cannot be made.

Using fixed force data to obtain firmness type curves, the possibly misleading results of such comparisons may be illustrated. The data used are indicated by the large dots on the family of curves of Fig. 6. By dividing the fixed force value in grams by the deformation in mm. a quantity is obtained, the dimensions of which are grams/mm., the same as for firmness readings.

Firmness type curves based on those data are indicated by broken lines and, obviously, do not correspond in profile to the curve produced

by the fixed strain technique, as represented by the solid curve. As the data represent one sample of bread and the curves indicate three differing rates of change of firmness, it is evident that data obtained by fixed force technique cannot be compared with those obtained by fixed deformation methods by the simple use of reciprocals. It is further indicated that small changes in fixed force value alter the nature of the resulting curves appreciably.

In tests of crumb at 18 and 66 hours, it is desirable that a deformation be used which will indicate a maximum change of required force over the test period. Fig. 8 represents the firmness of bread crumb

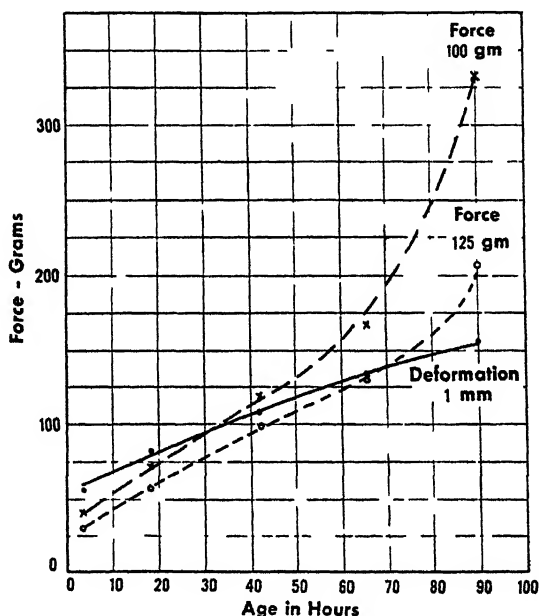


Fig. 7. Contrasting profiles of time-firmness curves obtained by fixed deformation technique and those calculated from fixed force data on the same bread sample. The captions on the curves indicate the fixed condition under which the data were obtained.

prisms at 66 hours minus the firmness at 18 hours at deformations up to 6 mm.

Up to a deformation of 1.5 mm., the slope of the curve is quite high. Evidently slight variations in strain value at which force is read would cause considerable fluctuations in the results. As the Baker machine is normally read while running, it appears inadvisable to take readings at less than 1.5 mm. It will be noted that the difference increases very little for deformations over 3 mm. There is no readily apparent way of selecting any particular value over 1.5 mm. that would be more valid than another. However, the use of lower deformations allows a

study of products over longer intervals of time because off-scale values are produced at a later time. Based on these considerations, a deformation of 2.5 mm. was selected as optimum.

*Suggested Method for Determination of Staling Rate of Bread with the Baker Compressimeter.* Select two or more loaves of comparable volumes from each batch to be tested. Wrap in moisture-proof paper and store at 86°F. At 18 hours from the oven, using commercial slice or 12 mm. slices, cut prisms to the dimensions of the plunger (32 mm. square). The prism should be taken at about 1 inch from the lower crust and the side away from the "break." Use half of each loaf, discarding those prisms with large holes or hard cores. The two end

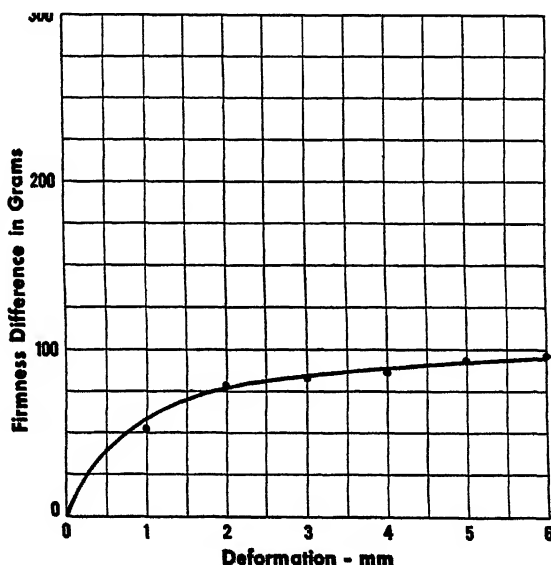


Fig. 8. The influence of deformation on the firmness-difference of bread crumb 66 and 18 hours old.

slices should be used to cap the open end of the loaf before rewrapping and returning to storage.

With the plunger at position 1, place the test prism beneath the plunger and turn Nut B (1) to adjust the pointers to zero. Start the motor and when the test piece has been compressed 2.5 mm., read scale J and multiply by the leverage. Average all readings for the batch.

At 66 hours from the oven, test the remaining halves in the same manner. The difference between the 3rd and 1st day averages is an index of the staling rate of the batch.

*Correlation of Test Method with Consumer Evaluations.* To determine whether the values measured by such a test would correlate with

consumer evaluations of firmness, bread 1, 2, and 3 days old was tested for firmness, as described, and, then, slices of each were offered to a panel of 20 people. Each was asked, "Which is most firm?" and then, "Which of the two others is less firm?"

Of the 20 people, 18 judged the 3 day old bread with a firmness reading of 195 grams as most firm and 18 judged the 1 day old bread with a reading of 105 grams as least firm. The two day old bread gave a reading of 131 grams.

These results show that the measurement of firmness by the Baker Compressimeter, using the selected test conditions, correlates with normal consumer evaluation of firmness.

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# CHANGES IN THE RATE OF FIRMNESS DEVELOPMENT IN BREAD AT DIFFERENT SEASONS AND WITH THE USE OF EMULSIFIERS<sup>1</sup>

OSCAR SKOVHOLT and R. L. DOWDLE<sup>2</sup>

## ABSTRACT

Compressibility determinations indicated that emulsifiers reduce the rate at which bread increases in firmness with age and that polyoxyethylene stearate is more effective than a vegetable oil monoglyceride in this respect. Measurements made during different seasons of the year showed that bread became firm more quickly in temperatures such as prevail indoors during winter as compared with those encountered during the warmer months of summer.

A controversy exists as to whether some of the recently developed emulsifiers are in effect merely bread softeners or whether they retard the rate of firmness development. The term "staling" might be applied to the latter change since it is believed that this phenomenon is chiefly characterized by increasing firmness and harshness when eaten or when judged by some type of squeeze test. Much work remains to be done in defining the process of staling. Until such a complete definition is available, controversy can be avoided by referring to the observed changes in firmness with age rather than to antistaling properties.

The purpose of this investigation was to determine the effect of two different types of emulsifiers on the rate of firmness development in bread. There was no change in the absorption used since extra water tends to increase the initial softness. Loaf volumes were approximately the same throughout the investigation. Variation in volume can also be an important factor in variable initial softness. The amount of bake also influences initial softness and this factor was kept constant throughout the investigation. An effect of temperature upon rate of firmness development became evident while making these compressibility measurements throughout the year. Some average values were obtained for the rate of firmness development during different seasons of the year.

## Materials and Methods

In order to conform to commercial baking practice, a sponge and dough method with average commercial formula was employed. This

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<sup>2</sup> From the laboratories of Quality Bakers of America Cooperative, Inc., N. Y., N. Y.

included the use of 3% shortening in the control doughs and 2% when using an emulsifier. Loaves were produced in pans with dimensions of  $4 \times 8 \times 2\frac{1}{2}$  in. and with doughs scaled at 430 g. Time and temperature factors for all steps in production were in line with accepted commercial practice. A baking time of 24 min. at 230°C. produced a more thorough bake than is evident in much commercial bread at the present time.

Two types of flour were used throughout the investigation but with a different sample for each set of baking tests. The flours used were average bakers southwestern hard winter and hard spring wheat types of medium extraction. Each set of loaves compared for firmness was made with either the same flour or a blend of similar samples.

Bread firmness was determined by the Baker Compressimeter using the optional method of constant strain as mentioned in Cereal Laboratory Methods (1). Readings were taken of the load required for depressions of 2, 3, 4 and 5 mm. These readings were made on at least six slices from each loaf tested.

Loaves were wrapped in ordinary waxed paper as soon as sufficiently cooled. They were then held in a cabinet and were subject to the usual variations in temperature until they were used for compressibility tests.

Two types of emulsifiers were used in the series specifically set up to study the rate of firmness development. They were a polyoxyethylene stearate and a vegetable oil monoglyceride and both were used at a level of 0.5% of flour weight. The latter had a somewhat lower melting point (46°C.) than most ingredients first offered the bakers as glyceryl monostearate.

## Results and Discussion

*Effect of Emulsifiers.* The results in Fig. 1 show compressibility readings made on bread when 2, 24, 48, 72, and 96 hours old. Six slices were taken from a separate loaf from each batch for the firmness determinations at each time interval. Triplicate sets of tests were made with each flour type so that each point on the curves is based on 18 readings.

It is quite evident that differences in softness of the 2 hour bread are insignificant and that there is an appreciable decrease in the rate of firmness development due to the addition of the emulsifiers. The average firmness readings for both flour types on 96 hour bread were 29.4 for the control loaves, 22.6 when using vegetable oil monoglyceride and 16.5 for the bread containing polyoxyethylene stearate. The two flour types are not identified in the figure since the tests with

flour No. 1 were made several weeks before those with No. 2 and with an increase in average room temperatures during this interval. This difference in temperature during storage of the bread could account for the difference in the rate of firmness development with the two flour types as will be discussed later.

There has been some dispute about the amount of deformation which should be employed in making firmness tests with the Baker Compressimeter and also as to whether the data conform to Hooke's

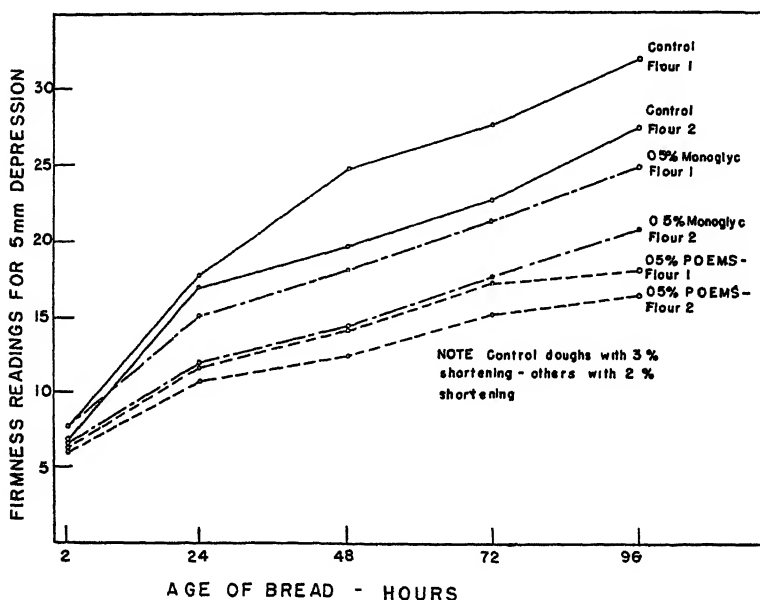


FIG. 1. Effect of age of bread on compressibility readings.

law. Fig. 2(b) shows the average firmness-deformation relationship of the control bread at various ages from the six series of tests which have just been discussed. Fig. 2(a) gives similar data for the bread containing polyoxyethylene stearate. The stress-strain relationships produced approximately straight lines in all cases when plotting deformations of 2, 3, 4, and 5 mm., but these lines extrapolated would not pass through the origin. This would seem to indicate that Hooke's law does not apply to such determinations. The regularity of results at different deformation levels show that the effect of the emulsifiers upon the rate of bread firmness development can be shown by employing any deformation within this range but that differences are greater in the upper part of the range since the lines are not parallel.

*Effect of Temperature.* One of the most impressive results from the use of an instrument such as the Baker Compressimeter was the revelation of a pronounced effect of storage temperature upon the rate of firmness development in bread. When judgment is used, instead of mechanical measurement, there is no way of noting whether bread firms more rapidly during the winter as compared with the summer season. It was soon discovered that shorter storage periods had to be employed during the cold weather season to stay on the scale of the instrument when reading the load needed for a 5 mm. depression of the slice.

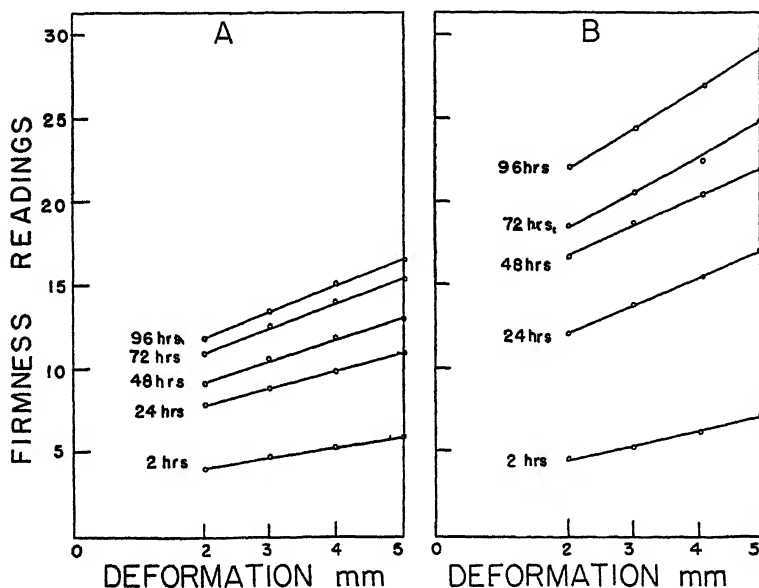


FIG. 2 (A) Relationship of firmness and deformation values obtained with bread of various ages containing polyoxyethylene stearate. (B) Values obtained with control bread of various ages

A total of 13 pairs of loaves made without emulsifier and a similar number containing polyoxyethylene stearate, at a level of 0.5% of flour weight, were checked for compressibility after four days storage at uncontrolled room temperatures during the months of May through August. Average compressibility values for a 5 mm. depression were 24.3 for the control loaves and 15.4 when the bread contained the emulsifier. By way of comparison, averages were compiled for 18 series of tests made during the months of October through April on bread which had been held for only 3 days. Readings for a 5 mm. depression averaged 30.1 for the control bread and 20.6 when the polyoxyethylene stearate was included. These values average 27%

higher than those obtained in the summer with a 25% reduction in storage time.

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### EFFECTS OF VARYING QUANTITIES OF SUGAR, SHORTENING, AND AMMONIUM BICARBONATE ON THE SPREADING AND TOP GRAIN OF SUGAR-SNAP COOKIES<sup>1</sup>

K. F. FINNEY, W. T. YAMAZAKI, and V. H. MORRIS<sup>2</sup>

#### ABSTRACT

Shortening was varied in the cookie formula from 25% to 35%, sugar from 50% to 80%, and ammonium bicarbonate from 0% to 3.25%, using a procedure that required small amounts of ingredients. Varying the quantity of shortening did not materially affect cookie diameter but did alter top grain in certain instances. Spreading of cookies during baking was directly proportional to the quantity of sugar added within each ammonium bicarbonate concentration. Increases in ammonium bicarbonate also produced proportional increases in diameter within each sugar level. When the quantity of sugar used was less than 55%, however, inferior top grain was obtained. An addition of .5% ammonium bicarbonate, in general, produced as much increase in cookie diameter and change in top grain as an increase of 7.6% sugar.

The quantity of ammonium bicarbonate required to produce cookies having a specified diameter and optimum top grain differed markedly between varieties. The flours from wheat varieties that spread the most with a fixed concentration of ammonium bicarbonate were those generally considered to be superior for cookies. Increasing increments of ammonium bicarbonate increased the pH of cookie dough from about 7.3 with no ammonium bicarbonate to about 7.9 for 2.25% regardless of variety. Further increases of bicarbonate to 3.25% were without additional effect on pH.

A formula including those quantities of sugar, shortening, and ammonium bicarbonate found to be most satisfactory for evaluating the cookie quality of different varieties of wheat is suggested.

The Tentative A.A.C.C. Cookie Method has been modified and adapted to a micro technique (1) for the quality characterization of soft wheat varieties. Before adopting this micro procedure for ex-

<sup>1</sup> Manuscript received September 30, 1949. Cooperative investigation between the Division of Cereal Crops and Diseases, Bureau of Plant Industry, Soils, and Agricultural Engineering, Agricultural Research Administration, U. S. Department of Agriculture, and Department of Agronomy, Ohio Agricultural Experiment Station.

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tended routine use in the Soft Wheat Laboratory, however, it seemed desirable to study the effect of varying quantities of the ingredients most effective in altering cookie characteristics. This paper presents data showing the relation of varying quantities of each of the ingredients sugar, shortening, and ammonium bicarbonate to spreading, thickness, and changes in top grain of sugar-snap cookies.

### Materials and Methods

The flour referred to as "cookie standard" and used in studying the effect of varying quantities of sugar, shortening, and ammonium bicarbonate on cookie properties was made up by compositing small quantities of flour milled on the Buhler experimental mill. It consisted of a composite of many samples of the better cookie baking varieties grown in 1944. Micro method I previously described in a companion paper (1) was used for baking cookies, except that shortening content was varied from 25% to 35%, sugar from 50% to 80%, and ammonium bicarbonate from 0 to 3.75%.

Six flours were used in studies on the response of typical wheat flours to varying quantities of ammonium bicarbonate. They were experimentally milled from six wheat varieties, each of which was a composite of grain grown at widely different locations in the eastern states in 1943 and 1944. The varieties consisted of four soft red winters, Thorne, Trumbull, Wabash, and Kawvale, the latter being generally considered of poor soft wheat quality; one soft white winter, American Banner; and one hard red winter, Purkof. Micro method I with the fixed sugar/shortening ratio of 60/30 was employed for baking cookies from these varieties. The amount of ammonium bicarbonate was varied from 0 to 3.75%.

Baking was carried out at 400°F. for 9 to 10 minutes, depending on the amount of spreading. Diameter (D) was measured on all cookies. Thickness (T) and spread factor (D/T) were determined on all cookies excepting certain of them containing high sugar contents in combination with high ammonium bicarbonate levels. Along the rims of these latter cookies there was usually a ridge that interfered with making thickness measurements. These cookies also became brittle shortly after cooling, and were not considered to be palatable.

### Results

*Effect of Varying Quantities of Sugar, Shortening, and Ammonium Bicarbonate.* Cookie baking data obtained in the studies with varying amounts of sugar, shortening, and ammonium bicarbonate, using the cookie standard flour, are summarized in Table I and presented graphically or photographically in Figs. 1 to 5.

TABLE I  
DIAMETER, THICKNESS, AND SPREAD FACTOR OF COOKIES BAKED WITH  
VARYING SUGAR/SHORTENING RATIOS AT EACH OF SEVERAL  
AMMONIUM BICARBONATE LEVELS

| NH <sub>4</sub> HCO <sub>3</sub> | Diam-eter (D)      | Thick-ness (T) | D/T  | Diam-eter (D) | Thick-ness (T) | D/T  | Diam-eter (D) | Thick-ness (T) | D/T  | Diam-eter (D)      | Thick-ness (T) | D/T                |
|----------------------------------|--------------------|----------------|------|---------------|----------------|------|---------------|----------------|------|--------------------|----------------|--------------------|
|                                  | cm.                | cm.            |      | cm.           | cm.            |      | cm.           | cm.            |      | cm.                | cm.            |                    |
|                                  | 50/30 <sup>1</sup> |                |      | 55/30         |                |      | 60/25         |                |      | 60/30              |                |                    |
| 0.00                             | 7.81               | 1.00           | 7.8  | 7.92          | 0.96           | 8.3  | 8.18          | 0.90           | 9.2  | 8.14               | 0.85           | 9.7                |
| 0.25                             | 7.84               | .98            | 8.1  | 8.01          | .88            | 9.4  | 8.34          | .87            | 9.7  | 8.35               | .82            | 10.3               |
| 0.75                             | 8.05               | .91            | 8.9  | 8.43          | .81            | 10.5 | 8.67          | .78            | 11.1 | 8.59               | .77            | 11.2               |
| 1.25                             | 8.42               | .83            | 10.2 | 8.85          | .74            | 12.0 | 9.13          | .72            | 12.8 | 8.94               | .71            | 12.7               |
| 1.75                             | 8.79               | .79            | 11.2 | 9.30          | .68            | 13.7 | 9.74          | .65            | 15.2 | 9.34               | .67            | 14.1               |
| 2.25                             | 8.95               | .80            | 11.3 | 9.56          | .63            | 15.4 | 10.13         | .58            | 17.7 | 9.87               | .58            | 17.3               |
| 2.75                             | 9.32               | .74            | 12.7 | 9.96          | .55            | 18.1 | 10.34         | —              | —    | 10.12              | .54            | 18.9               |
| 3.25                             | 9.76               | .65            | 15.1 | 10.08         | .53            | 19.1 | 10.49         | —              | —    | 10.46              | .53            | 20.0               |
|                                  | 60/35              |                |      | 65/25         |                |      | 65/30         |                |      | 65/35              |                |                    |
| 0.00                             | 8.39               | 0.89           | 10.7 | 8.27          | 0.89           | 9.4  | 8.47          | 0.79           | 10.8 | 8.46               | 0.82           | 10.7               |
| 0.25                             | 8.69               | .72            | 12.2 | 8.44          | .80            | 10.6 | 8.66          | .76            | 11.4 | 8.66               | .76            | 11.5               |
| 0.75                             | 8.89               | .70            | 12.8 | 8.92          | .72            | 12.6 | 9.01          | .66            | 13.7 | 8.83               | .70            | 12.7               |
| 1.25                             | 9.28               | .62            | 15.0 | 9.35          | .63            | 14.9 | 9.56          | .57            | 17.1 | 9.24               | .66            | 14.1               |
| 1.75                             | 9.66               | .57            | 17.1 | 9.83          | .56            | 17.6 | 9.96          | .54            | 18.6 | 9.69               | .58            | 16.8               |
| 2.25                             | 10.10              | .52            | 19.7 | 10.10         | —              | —    | 10.28         | —              | —    | 10.04              | .53            | 18.9               |
| 2.75                             | 10.17              | .52            | 19.5 | 10.41         | —              | —    | 10.64         | —              | —    | 10.29              | —              | —                  |
| 3.25                             | 10.71              | .52            | 20.6 | 10.62         | —              | —    | 10.72         | —              | —    | 10.75              | —              | —                  |
|                                  | 70/25              |                |      | 70/30         |                |      | 70/35         |                |      | 75/30 <sup>2</sup> |                | 80/30 <sup>2</sup> |
| 0.00                             | 8.53               | 0.77           | 11.1 | 8.72          | 0.74           | 11.9 | 8.71          | 0.73           | 12.1 | 8.88               |                | 9.03               |
| 0.25                             | 8.80               | .71            | 12.4 | 8.78          | .75            | 11.7 | 8.92          | .65            | 13.8 | 8.90               |                | 9.18               |
| 0.75                             | 9.46               | —              | —    | 9.18          | .65            | 14.2 | 9.10          | —              | —    | 9.34               |                | 9.62               |
| 1.25                             | 9.95               | —              | —    | 9.52          | —              | —    | 9.50          | —              | —    | 9.85               |                | 10.29              |
| 1.75                             | 10.22              | —              | —    | 9.92          | —              | —    | 9.89          | —              | —    | 10.21              |                | 10.60              |
| 2.25                             | 10.60              | —              | —    | 10.39         | —              | —    | 10.20         | —              | —    | 10.67              |                | —                  |
| 2.75                             | —                  | —              | —    | 10.65         | —              | —    | —             | —              | —    | 10.86              |                | —                  |
| 3.25                             | —                  | —              | —    | 10.81         | —              | —    | —             | —              | —    | 10.95              |                | —                  |

<sup>1</sup> Ratio of sugar (%) to shortening (%).

<sup>2</sup> Diameters only were recorded because of ridges on the rim of each cookie.

The effects on cookie diameter of varying the shortening content at each of several ammonium bicarbonate levels are illustrated graphically in Fig. 1. Each point in Fig. 1 is the average of the data for all three sugar levels (Table I). Ammonium bicarbonate produced marked increases in cookie diameter whereas shortening had very little effect. Certain properties of the top grain (Fig. 2), however, are influenced by the ratio of shortening to sugar. For example, 25% shortening with 60% sugar resulted in well-defined top grains with a

rather undesirably hard and lean appearance. Increasing the shortening to 30% with 60% sugar resulted in cookies that had equally good top grains and were desirably softer and richer in appearance. When the shortening was increased to 35% with 60% sugar, dull and extremely soft appearing cookies with inferior top grains were obtained. Thus with 60% sugar in the formula, 30% shortening was preferable.

Increasing the sugar to 65% and 70% at the 25% shortening level resulted in top grains that were hard in appearance and more open than those with 60% sugar. These top grains for 65% and 70% sugar became closer and softer appearing, in general, as the shortening was increased.

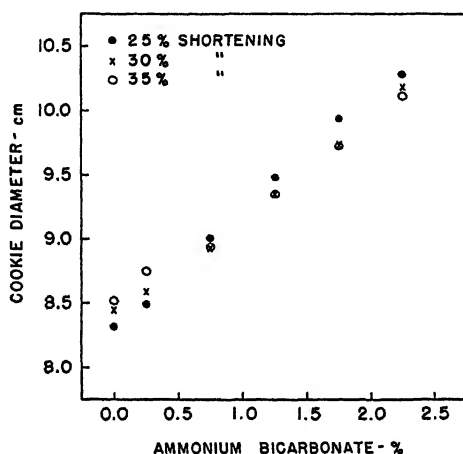


Fig. 1. Effect on cookie diameter of varying the shortening content from 25% to 35% at each of several ammonium bicarbonate levels.

When increased diameter and openness of top grain accompanying increases of ammonium bicarbonate are taken into account, equally good cookies were obtained with 30% shortening for all three sugar levels; whereas, the general appearance of the cookies containing 25% shortening was short of that desired. These observations together with the unsatisfactory top grains for 35% shortening with 60% sugar, and the necessity of increasing the quantity of sugar to overcome these undesirable top grains are factors that determined the choice of 30% shortening in all subsequent studies.

The effect on cookie diameter of varying quantities of ammonium bicarbonate and sugar in combination with a fixed shortening content of 30% is illustrated graphically in Fig. 3. Correlation coefficients and regression equations for the lines showing the relation between cookie diameter and quantity of ammonium bicarbonate within each



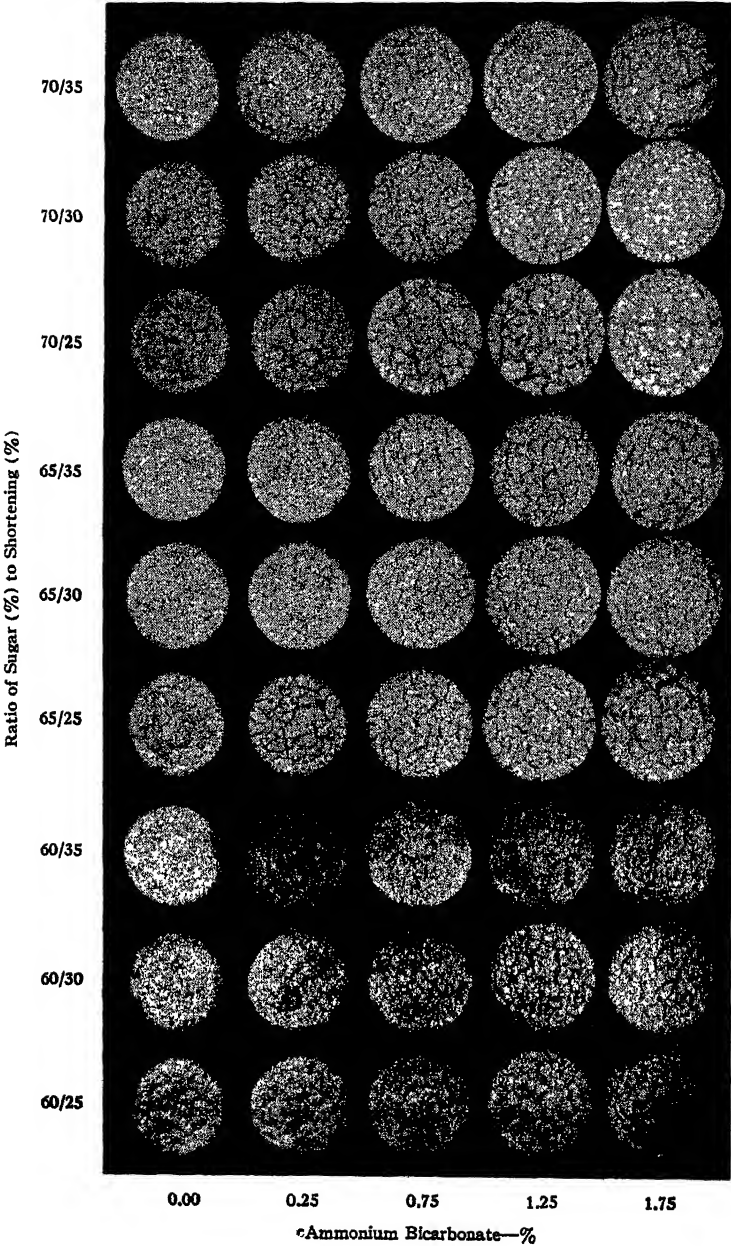


Fig. 2. Effect on cookie top grain of varying the ratio of sugar to shortening at each of several ammonium bicarbonate levels.

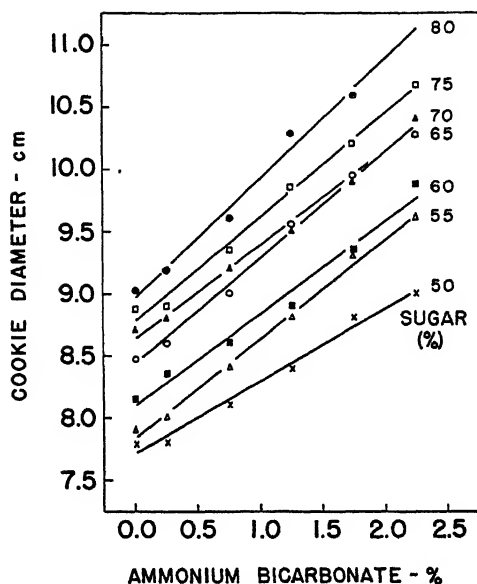


Fig. 3. Effect on cookie diameter of varying quantities of sugar and ammonium bicarbonate in combination with a fixed shortening content of 30%.

of seven sugar levels (50% to 80%) are given in Table II. Within each sugar level, the response to ammonium bicarbonate was linear over the range 0% to 2.25%. The average slope for all regression lines indicates that each .5% increment of ammonium bicarbonate increased cookie diameter by 3.9 mm.

As the concentration of sugar in the formula was increased from 50% to 80% within each ammonium bicarbonate level, cookie diameter increased. Referring to the cookie diameters calculated for a con-

TABLE II  
CORRELATION COEFFICIENTS AND REGRESSION EQUATIONS FOR COOKIE DIAMETER AND AMMONIUM BICARBONATE CONTENT FOR EACH OF SEVEN SUGAR LEVELS AT 30% SHORTENING

| Sugar content | $\text{NH}_4\text{HCO}_3$ levels (n) | Corr. coeff. (r)   | Regression equation $Y =$ | Calculated diameter at 0.75% $\text{NH}_4\text{HCO}_3$ (Y) |
|---------------|--------------------------------------|--------------------|---------------------------|------------------------------------------------------------|
| %             |                                      |                    |                           | cm.                                                        |
| 50            | 6                                    | 0.992 <sup>1</sup> | $0.577X + 7.715$          | 8.15                                                       |
| 55            | 6                                    | 0.997              | $0.791X + 7.843$          | 8.44                                                       |
| 60            | 6                                    | 0.986              | $0.738X + 8.098$          | 8.65                                                       |
| 65            | 6                                    | 0.994              | $0.852X + 8.446$          | 9.08                                                       |
| 70            | 6                                    | 0.996              | $0.748X + 8.638$          | 9.20                                                       |
| 75            | 6                                    | 0.992              | $0.838X + 8.777$          | 9.41                                                       |
| 80            | 5                                    | 0.993              | $0.959X + 8.973$          | 9.69                                                       |

<sup>1</sup> All correlation coefficients are significant beyond the 0.1% point.

centration of 75% ammonium bicarbonate (Table II), a 30% increase in sugar increases diameter by 15.4 mm (96.9 mm minus 81.5 mm) and 1% results in an increase of 0.51 mm. Thus an increase of 7.6% sugar (3.9 – 51) in the formula produces as much spreading as a 0.5% increment of ammonium bicarbonate.

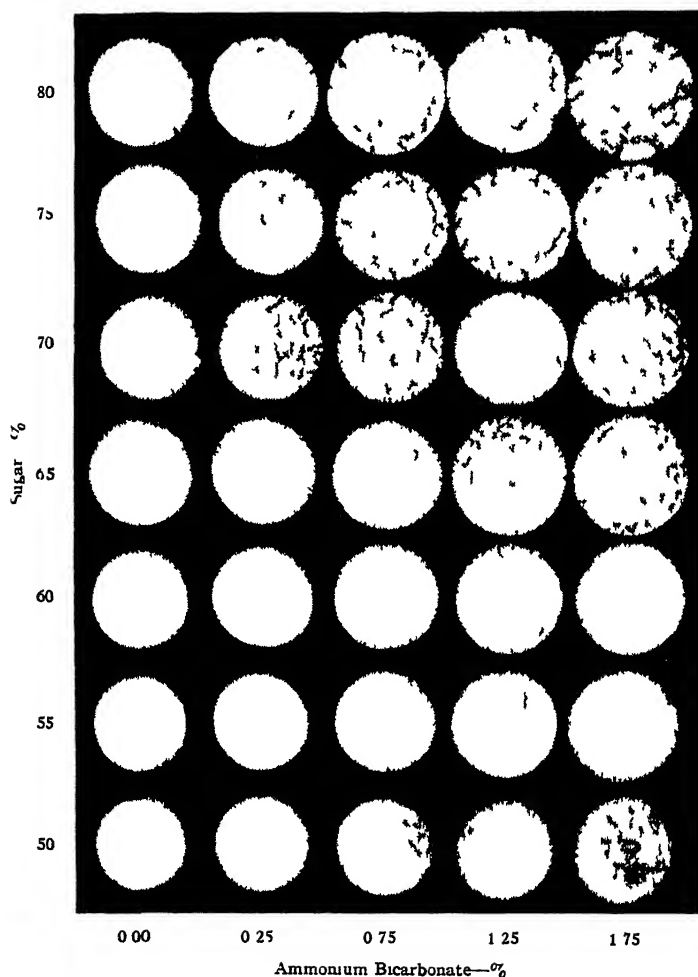


Fig 4 Top grain and relative diameter of representative cookies containing varying quantities of sugar and ammonium bicarbonate

The appearance of representative cookies containing varying quantities of sugar and ammonium bicarbonate is shown in Fig. 4. The effect of these two ingredients on top grain of cookies is of particular interest. The optimum top grains at or between the 1.25 and 1.75%

ammonium bicarbonate levels for the 55% sugar level are or would be (interpolating) slightly poorer than for higher sugar levels; and there is no satisfactory top grain for the 50% sugar level. These results suggest that 55% sugar is the minimum quantity that should be used in this cookie formula if satisfactory top grain is to be obtained. The cookie (Fig. 4) containing 60% sugar and 0.75% ammonium bicarbonate is one of the several examples of approximately optimum top grain. An optimum top grain for the 60% sugar level would be expected with about 1% ammonium bicarbonate. The cracks or valleys produced with 1.25% are considered to be too wide.

As was true for diameter, the width of the cracks increases as ammonium bicarbonate increases within each sugar level, and as sugar

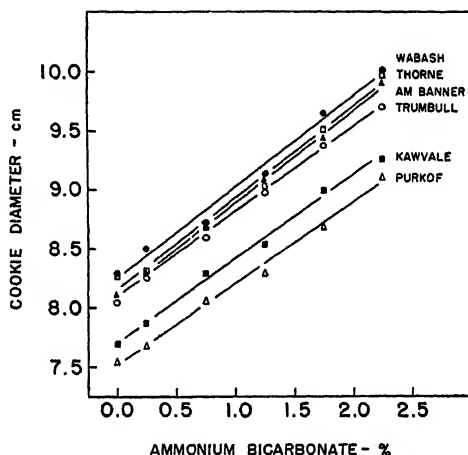


Fig. 5. Effect of varying quantities of ammonium bicarbonate on the cookie diameter of wheat varieties.

content increases within each ammonium bicarbonate level. Thus, for the flour used in this phase of the work, a cookie with a diameter of about 8.8 cm. and a satisfactory top grain was produced with the formula containing 70% sugar and .25% ammonium bicarbonate (Figs. 3 and 4). Interpolating in Fig. 3, a cookie with the same diameter and equally good top grain also can be produced with 10% less sugar providing the ammonium bicarbonate is increased to 1%. Similarly a cookie with a diameter of about 9 cm and a desirable top grain was produced with 80% sugar and no ammonium bicarbonate. A cookie of equal diameter and top grain also was produced with 15% less sugar when .75% ammonium bicarbonate was added.

*Response of Wheat Varieties to Varying Quantities of Ammonium Bicarbonate.* Wheat flours from six varieties were baked into cookies

at each of several ammonium bicarbonate levels for the purpose (1) of testing further the formula containing 60% sugar and 30% shortening, (2) of establishing the most satisfactory level of ammonium bicarbonate to use in the baking formula, and (3) of determining whether there are varietal differences in ammonium bicarbonate requirement.

TABLE III

DIAMETER AND THICKNESS OF COOKIES BAKED FROM SIX WHEAT VARIETIES AT EACH OF SEVERAL AMMONIUM BICARBONATE LEVELS TOGETHER WITH THE pH OF EACH COOKIE DOUGH

| NH <sub>4</sub> HCO <sub>3</sub> | Diameter | Thickness | pH   | Diameter   | Thickness | pH   |
|----------------------------------|----------|-----------|------|------------|-----------|------|
| %                                | cm.      | cm.       |      | cm.        | cm.       |      |
| PURKOF                           |          |           |      | WABASH     |           |      |
| 0.00                             | 7.54     | 1.05      | 7.40 | 8.29       | 0.84      | 7.24 |
| 0.25                             | 7.67     | 0.98      | 7.61 | 8.50       | .78       | 7.50 |
| 0.75                             | 8.06     | .90       | 7.61 | 8.72       | .74       | 7.60 |
| 1.25                             | 8.29     | .84       | 7.76 | 9.13       | .68       | 7.80 |
| 1.75                             | 8.68     | .79       | 7.85 | 9.65       | .61       | 7.77 |
| 2.25                             | 9.10     | .73       | 7.92 | 10.01      | .56       | 7.86 |
| 2.75                             | 9.21     | .71       | 7.90 | 10.63      | .48       | 7.84 |
| 3.25                             | 9.61     | .64       | 7.92 | 10.94      | .45       | 7.91 |
| 3.75                             | 9.71     | .60       | 7.89 | 11.40      | .45       | 7.86 |
| KAWALE                           |          |           |      | TRUMBULL   |           |      |
| 0.00                             | 7.70     | .94       | 7.41 | 8.05       | .88       | 7.19 |
| 0.25                             | 7.87     | .92       | 7.55 | 8.25       | .87       | 7.49 |
| 0.75                             | 8.29     | .86       | 7.67 | 8.59       | .79       | 7.67 |
| 1.25                             | 8.54     | .81       | 7.77 | 8.97       | .74       | 7.68 |
| 1.75                             | 8.99     | .73       | 7.89 | 9.37       | .67       | 7.82 |
| 2.25                             | 9.26     | .69       | 7.90 | 9.70       | .60       | 7.88 |
| 2.75                             | 9.67     | .63       | 7.87 | —          | —         | —    |
| 3.25                             | 10.01    | .52       | 7.81 | —          | —         | —    |
| THORNE                           |          |           |      | AM. BANNER |           |      |
| 0.00                             | 8.27     | .86       | 7.33 | 8.12       | .87       | 7.27 |
| 0.25                             | 8.32     | .84       | 7.39 | 8.30       | .83       | 7.55 |
| 0.75                             | 8.69     | .77       | 7.68 | 8.70       | .79       | 7.65 |
| 1.25                             | 9.03     | .72       | 7.81 | 9.08       | .71       | 7.82 |
| 1.75                             | 9.51     | .65       | 7.89 | 9.43       | .64       | 7.80 |
| 2.25                             | 9.97     | .59       | 7.93 | 9.90       | .60       | 7.88 |

These studies were concerned with the characterization and evaluation of flours recognized as having markedly different properties that make them of variable value for the production of soft wheat bakery products. The results are summarized in Table III and presented graphically or photographically in Figs. 5, 6, and 7. Correlation coefficients and regression equations for the lines showing the relation

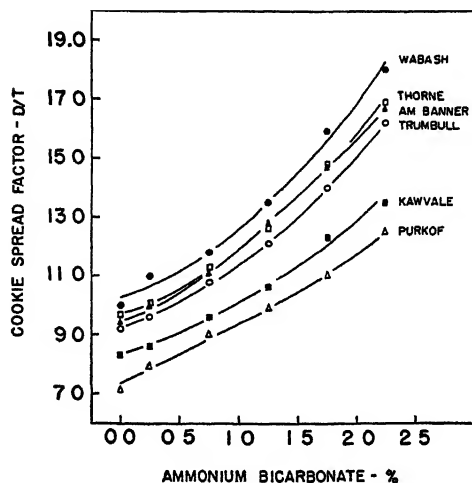


Fig 6. Effect of varying quantities of ammonium bicarbonate on the cookie spread factor (D/T) of wheat varieties.

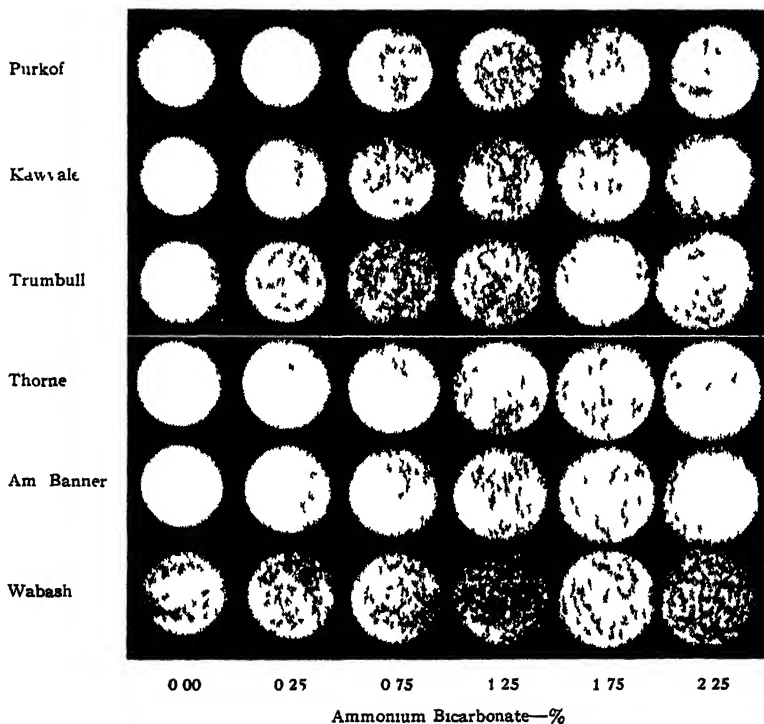


Fig 7 Effect of varying quantities of ammonium bicarbonate on the top grain and relative diameter of cookies baked from Purkof, Kawvale, Trumbull, Thorne American Banner and Wabash wheats

between cookie diameter and ammonium bicarbonate with each of the six varieties are given in Table IV.

Data in Fig. 5 and correlation coefficients in Table IV show that cookie diameter within a variety is a linear function of the quantity of ammonium bicarbonate used. These data and conclusions are in agreement with those obtained for the "cookie standard" flour. For *spread factor* the relationship is curvilinear (Fig. 6), the result of dividing an increasing number by one that is decreasing. These data indicate that in the routine characterization and evaluation of experimental and commercial varieties of wheat, the concentration of ammonium bicarbonate used in the formula is not critical. It was observed, however, that contraction during cooling of the cookies containing the higher bicarbonate concentrations was not as consistent as for the lower concentrations, thereby causing surface irregularities that introduced errors in measuring thickness.

TABLE IV  
CORRELATION COEFFICIENTS AND REGRESSION EQUATIONS FOR COOKIE  
DIAMETER AND AMMONIUM BICARBONATE CONTENT FOR  
EACH OF SIX VARIETIES OF WHEAT

| Variety    | NH <sub>4</sub> HCO <sub>3</sub><br>levels<br>(n) | Corr. coeff<br>(r) | Regression equation<br>Y = | Calculated diam-<br>eter at 0.75%<br>NH <sub>4</sub> HCO <sub>3</sub><br>(Y) |
|------------|---------------------------------------------------|--------------------|----------------------------|------------------------------------------------------------------------------|
|            |                                                   |                    |                            | cm.                                                                          |
| Wabash     | 6                                                 | 0.990 <sup>1</sup> | 0.772X+8.246               | 8.82                                                                         |
| Thorne     | 6                                                 | 0.989              | 0.771X+8.163               | 8.74                                                                         |
| Am. Banner | 6                                                 | 0.998              | 0.781X+8.103               | 8.69                                                                         |
| Trumbull   | 6                                                 | 0.999              | 0.720X+8.100               | 8.64                                                                         |
| Kawvale    | 6                                                 | 0.995              | 0.706X+7.714               | 8.24                                                                         |
| Purkof     | 6                                                 | 0.996              | 0.688X+7.517               | 8.03                                                                         |

<sup>1</sup> All correlation coefficients are significant beyond the 0.1% point

The extent to which the six varieties differ as to the quantity of ammonium bicarbonate required to produce cookies of equal diameter or spread factor is strikingly illustrated in Figs. 5 and 6. For example in Fig. 5, a rule placed horizontally across the regression lines at a diameter of 9 cm. indicates that Wabash requires slightly more than 0.75%, Thorne and American Banner slightly more than 1%, Trumbull about 1.25%, Kawvale about 1.75%, and Purkof somewhat more than 2% ammonium bicarbonate to produce a cookie with a diameter of 9 cm. The quantities of ammonium bicarbonate required to produce equally good top grains for all six varieties (Fig. 7) are in very close agreement with those required to give a diameter of 9 cm. (Fig. 5 and Table III). Thus Purkof required about 170% more ammonium bicarbonate than did Wabash to produce a cookie of equal diameter and top grain.

*Effect of Ammonium Bicarbonate Concentration on Dough pH.* In order to determine whether there was a close relationship between the quantity of ammonium bicarbonate in the formula and the reaction of the cookie dough, the pH of each dough in the experiment discussed above was measured as it came out of the mixer. The results are summarized in Table III. These data show that doughs without ammonium bicarbonate have a pH of about 7.3; increasing quantities of bicarbonate gradually increase the pH, until with 2.25% ammonium bicarbonate, a pH of about 7.9 is reached. Further additions of the base, however, have no effect on the reaction. Thus the greater capacity of some varieties to spread with a fixed quantity of ammonium bicarbonate and the differences in bicarbonate requirement between varieties do not appear to be attributable to a pH effect.

### Discussion

It appears that within reasonable limits, the proportions of sugar, shortening and ammonium bicarbonate are not critical in a cookie test baking formula from the standpoint of characterizing and differentiating varieties. The important consideration is to choose a formula which will produce normal, well-shaped cookies from flours representing a wide range of quality. Such a formula should produce cookies having large diameters, flat tops, and well developed cracks with flours from soft varieties, and cookies having much smaller diameters and a minimum of top grain from semihard to hard varieties. A formula containing 60% sugar, 30% shortening and 0.75% ammonium bicarbonate met these requirements with the flours tested.

Desirable diameters and top grains were obtained from flours generally considered to be of poor cookie quality (Purkof and Kawvale) by increasing the ammonium bicarbonate in the formula. Equal diameters and as good or better top grains were produced from flours generally considered of good cookie quality with *less sugar* and the same concentration of ammonium bicarbonate required by flours of poor and medium cookie quality.

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# MICRO VERSUS MACRO COOKIE BAKING PROCEDURES FOR EVALUATING THE COOKIE QUALITY OF WHEAT VARIETIES<sup>1</sup>

K. F. FINNEY, V. H. MORRIS, and W. T. YAMAZAKI<sup>2</sup>

## ABSTRACT

Cookie baking equipment and techniques suitable for evaluating the quality of 40 g. of flour are described. Cookie baking data for the "Tentative A.A.C.C." macro cookie test and three micro procedures indicate that all four methods are equally satisfactory for evaluating the cookie baking potentialities of wheat flours. With respect to the saving of time and ingredients, however, the micro procedures are markedly superior to the macro test from which they were derived. There is considerable latitude with respect to the type of mixing equipment that may be used for the preparation of cookie doughs, providing the mixing of the flour with the other creamed ingredients is held to a minimum.

The cookie baking formula and procedure originally proposed by Alexander (1) was modified by the 1941-42 Biscuit and Cracker Flours Committee (3) and recommended for the evaluation of soft wheat flours. This and other collaborative studies have resulted in a recommended formula and procedure called the "Tentative Cookie Method." The use of this test, however, in the quality research and variety characterization program of the Federal Soft Wheat Laboratory has been limited because of the relatively large quantity of flour required. Accordingly, it has been modified<sup>3</sup> and adapted to micro techniques for evaluating the cookie quality of small lots of flour obtained from strains developed in the wheat breeding programs. This paper describes the equipment and techniques of these micro procedures, and compares them to the A.A.C.C. tentative procedure for evaluating typical varieties recognized for their divergent potentialities as cookie flours.

## Materials and Methods

The flours used were experimentally milled on the Buhler mill from twelve named varieties of wheat, each of which was a composite of

<sup>1</sup> Manuscript received September 30, 1949. Cooperative investigation between the Division of Cereal Crops and Diseases, Bureau of Plant Industry, Soils, and Agricultural Engineering, Agricultural Research Administration, U. S. Department of Agriculture, and Department of Agronomy, Ohio Agricultural Experiment Station.

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<sup>3</sup> See companion manuscript, "Effects of varying quantities of sugar, shortening, and ammonium bicarbonate on the spreading and top grain of sugar-snap cookies."

grain grown in the eastern uniform nurseries<sup>4</sup> at eight stations in 1945. Two other flour composites were included, namely (1) a composite of all the variety composites and (2) a blend of flour from several good quality soft wheat varieties referred to as "cookie standard." Protein, ash, and cookie dough absorption of each flour is given in Table I.

The formula and quantity of each ingredient used in the "Tentative A.A.C.C." macro test and the three micro procedures are given in Table II.

For the Tentative A.A.C.C. macro procedure, nonfat milk solids were added as a 20% suspension, 33.75 ml. of which contained 6.75 g. of solids. When nonfat milk solids were added as a suspension in any

TABLE I  
PROTEIN, ASH, AND COOKIE DOUGH ABSORPTION OF 14  
FLOURS THAT WERE BAKED INTO COOKIES<sup>1</sup>

| Sample          | Protein | Ash  | Absorption |
|-----------------|---------|------|------------|
|                 | %       | %    | %          |
| Cookie standard | 9.8     | 0.41 | 21.5       |
| Clarkan         | 9.2     | .42  | 22.5       |
| Minturki        | 8.8     | .42  | 22.3       |
| Kawvale         | 8.2     | .43  | 22.5       |
| Kharkof         | 8.9     | .43  | 22.8       |
| American Banner | 8.1     | .42  | 23.0       |
| Trumbull        | 8.7     | .42  | 22.8       |
| Purkof          | 8.9     | .42  | 22.8       |
| Fairfield       | 7.9     | .42  | 21.8       |
| Wabash          | 8.7     | .42  | 22.5       |
| Blackhawk       | 9.8     | .42  | 21.8       |
| H. Q. × Kawvale | 9.4     | .42  | 22.0       |
| Thorne          | 9.0     | .42  | 22.3       |
| Composite       | 8.8     | .42  | 22.3       |

<sup>1</sup> on 14% moisture basis.

of the micro procedures, 6 ml. containing 1.2 g. of solids were used. Whipping 120 g. of nonfat milk solids into 528 ml. of water gave 600 ml. of the 20% suspension. Foam was removed by the vacuum pump method.

A Hobart mixer (Model C-10 equipped with a cake paddle and a 3-quart bowl) was used for all creaming and mixing operations in the tentative A.A.C.C. macro procedure. The Hobart mixer also was used in the micro procedures for preparing in one operation all of the first stage of creamed material required for one day of baking. The remaining creaming and mixing involved in the micro methods was carried out in a National-Swanson-Working nonrecording micromixer modified to give a head speed of 178 rpm, and to use a flanged and deeper bowl with a capacity of 25 g. to 50 g. of flour (Fig. 1).

<sup>4</sup> The grain for these studies was obtained from tests conducted in cooperation with the several State agricultural experiment stations in the Eastern United States.

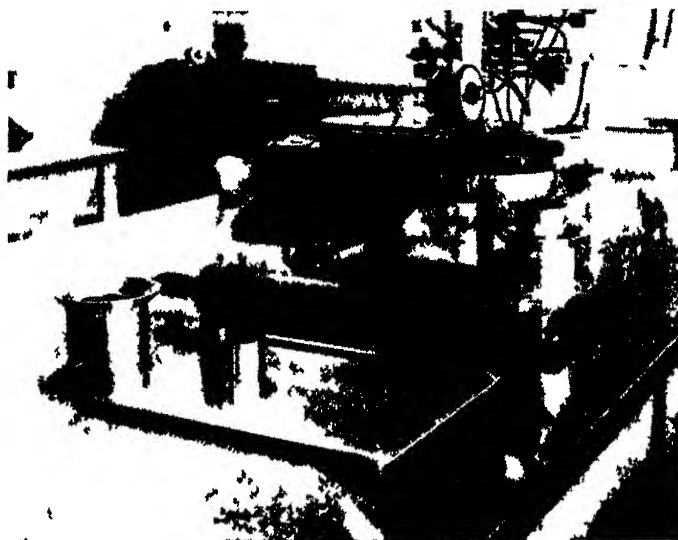


Fig 1 Mixer tray rolling pin and cutter used with the micro cookie procedures

The cookie cutter was made from stainless steel tubing having an inside diameter of 2 13/32 inches and thickness of 3/64 inch (18 gauge), was 1½ inches in height, and was tapered from the outside inward on one end to give a sharp and rigid cutting edge. The trays were sheet aluminum 13 inches × 10 inches × .094 inch thick with riders .275 inch high and .5 inch wide running lengthwise along both sides and attached with brass screws which were countersunk on the bottom side of the tray. The pieces of equipment that were modified or con-

TABLE II  
FORMULAE FOR THE A A C C MACRO AND THE THREE  
MICRO COOKIE BAKING PROCEDURES

| Ingredient                       | Cookie method |                    | Flour basis |
|----------------------------------|---------------|--------------------|-------------|
|                                  | A A C C macro | Micro I II and III |             |
|                                  | grams         | grams              | %           |
| Sugar                            | 135 00        | 24 00              | 60 00       |
| Shortening <sup>1</sup>          | 67 50         | 12 00              | 30 00       |
| NaHCO <sub>3</sub>               | 2 25          | 0 40               | 1 00        |
| NH <sub>4</sub> HCO <sub>3</sub> | 1 69          | 0 30               | 0 75        |
| Nonfat milk solids               | 6 75          | 1 20               | 3 00        |
| NaCl                             | 2.25          | 0 40               | 1.00        |
| Water                            | optimum       | optimum            | optimum     |
| Flour <sup>2</sup>               | 225 00        | 40 00              | 100 00      |

<sup>1</sup> Hydrogenated vegetable shortening especially prepared for biscuit and cracker manufacturing  
14% moisture

structed especially for the micro cookie procedures are shown photographically in Fig. 1.

Diameter (D) and thickness (T) were measured in millimeters and D/T was calculated for all cookies. Using a series of especially prepared cookies as reference standards, top grain was scored on the basis of 0 for a compact, hard-appearing cookie with no breaks, to 5 for a well-broken top containing numerous small "islands" characteristic of cookies baked from good quality cookie flours.

*Macro Method.* The tentative A.A.C.C. formula was modified slightly to conform with that used in the micro procedures, but in other respects the "macro" test as used in this study corresponds closely in important details to the Tentative A.A.C.C. test.

Sugar, shortening, and sodium bicarbonate were creamed for three minutes in a Hobart mixer on second speed, cutting down after each minute. Sodium chloride and ammonium bicarbonate were dissolved in the milk suspension and water in a 100 ml. beaker, after which the contents were added to the creamed mixture during mixing at low speed for one minute. Flour was incorporated by mixing for two minutes at low speed. After mixing, the dough was removed from the bowl with a spatula, care being taken to handle and compress the dough as little as possible. Doughs were spaced on trays, rolled once, and cut. That outside the stainless steel cutter was removed with a spatula before raising the cutter. Baking was carried out immediately for 10 minutes at 400°F. in a reel type oven.

*Micro Method I.* The first micro modification of the macro procedure involved the preparation of a sufficient quantity of sugar-shortening-sodium bicarbonate mixture for one day of baking by creaming 900 g. sugar, 450 g. shortening, and 15 g. sodium bicarbonate in the Hobart mixer for 4 minutes at high speed, scraping down at each half-minute interval. This creamed mass was sufficient for 37 weighings of 36.4 g. each on small squares of waxed paper.

Each cookie dough was prepared as follows: one 36.4 g. portion of the creamed mass of sugar, shortening, and sodium bicarbonate was transferred by means of a spatula to the micromixer bowl. The desired quantity of ammonium bicarbonate (.3 g.) was dissolved in 6 ml. of the milk suspension in a 10 ml. beaker, after which the contents were added to the bowl. The beaker was rinsed first with 2 ml. of sodium chloride solution containing 0.4 g. of salt and finally with 1.8 ml. to 3.3 ml. of water, depending on the requirement of the flour being tested. After creaming these liquids containing nonfat milk solids and sodium chloride for three minutes with the sugar-shortening-sodium bicarbonate creamed mixture, the flour was cut in with a spatula and then mixed for 10, 5, 5, and 5 seconds. The dough was dislodged with a

spatula from the bottom and sides of the bowl after each interval. After mixing, the dough was removed from the bowl and divided into two approximately equal parts with the aid of a spatula, care being taken to handle and compress the dough as little as possible. After spacing on a tray, the two doughs were rolled once with a wooden rolling pin and cut with a stainless steel cutter, dough outside the cutter being removed with a spatula before raising the cutter. Baking was carried out immediately for 10 minutes at 400°F. in a reel type oven.

*Micro Method II.* The second micro method differed from the first in that 15 g. of finely granulated sodium chloride and 45 g. of powered nonfat milk solids were creamed with the sugar, shortening,

TABLE III  
PROPERTIES OF COOKIES BAKED FROM 14 WHEAT VARIETIES  
BY A MACRO AND THREE MICRO PROCEDURES

| Sample                  | Diam-eter       | Thick-ness | D/T   | Top grain | Diam-eter        | Thick-ness | D/T   | Top grain |
|-------------------------|-----------------|------------|-------|-----------|------------------|------------|-------|-----------|
|                         | cm.             | cm.        |       |           | cm.              | cm.        |       |           |
|                         | MACRO METHOD    |            |       |           | MICRO METHOD I   |            |       |           |
| Standard                | 8.92            | 0.74       | 12.13 | 4         | 8.84             | 0.77       | 11.49 | 5         |
| Blackhawk               | 8.80            | .79        | 11.19 | 3         | 8.70             | .84        | 10.34 | 5         |
| Wabash                  | 8.72            | .81        | 10.72 | 4         | 8.58             | .84        | 10.24 | 5         |
| Harvest Queen × Kawvale | 8.70            | .78        | 11.11 | 3         | 8.54             | .84        | 10.16 | 4.5       |
| Fairfield               | 8.65            | .81        | 10.66 | 3.5       | 8.45             | .84        | 10.23 | 4.5       |
| Thorne                  | 8.62            | .84        | 10.30 | 4         | 8.52             | .84        | 10.20 | 4.5       |
| American Banner         | 8.61            | .84        | 10.28 | 4         | 8.43             | .87        | 9.66  | 4         |
| Kawvale                 | 8.52            | .85        | 9.98  | 3         | 8.20             | .91        | 9.06  | 4         |
| Clarkan                 | 8.50            | .86        | 9.99  | 2         | 8.28             | .88        | 9.43  | 3.5       |
| Composite               | 8.48            | .85        | 10.00 | 2.5       | 8.43             | .90        | 9.39  | 4         |
| Minturki                | 8.45            | .87        | 9.71  | 2.5       | 8.21             | .89        | 9.23  | 3.5       |
| Trumbull                | 8.31            | .89        | 9.34  | 3         | 8.10             | .95        | 8.55  | 3         |
| Kharkof                 | 8.17            | .94        | 8.75  | 1         | 7.85             | 1.00       | 7.83  | 2.5       |
| Purkof                  | 8.01            | .96        | 8.38  | 1         | 7.76             | 1.03       | 7.57  | 2.5       |
|                         | MICRO METHOD II |            |       |           | MICRO METHOD III |            |       |           |
| Standard                | 8.90            | 0.80       | 11.07 | 5         | 8.79             | 0.76       | 11.72 | 4.5       |
| Blackhawk               | 8.70            | .83        | 10.23 | 5         | 8.72             | .82        | 11.14 | 5         |
| Wabash                  | 8.69            | .85        | 10.27 | 4.5       | 8.63             | .83        | 10.74 | 5         |
| Harvest Queen × Kawvale | 8.50            | .86        | 9.92  | 4.5       | 8.50             | .84        | 10.32 | 4.5       |
| Fairfield               | 8.45            | .87        | 9.72  | 4.5       | 8.48             | .86        | 9.92  | 4.5       |
| Thorne                  | 8.49            | .88        | 9.70  | 4.5       | 8.39             | .86        | 9.82  | 5         |
| American Banner         | 8.36            | .89        | 9.45  | 4.5       | 8.40             | .87        | 9.75  | 4         |
| Kawvale                 | 8.22            | .91        | 9.05  | 4.5       | 8.34             | .87        | 9.73  | 3.5       |
| Clarkan                 | 8.28            | .91        | 9.08  | 4         | 8.35             | .89        | 9.53  | 4         |
| Composite               | 8.37            | .91        | 9.27  | 4.5       | 8.22             | .87        | 9.42  | 3         |
| Minturki                | 8.34            | .90        | 9.23  | 4         | 8.29             | .89        | 9.55  | 3.5       |
| Trumbull                | 8.22            | .95        | 8.63  | 3.5       | 8.17             | .93        | 8.93  | 4         |
| Kharkof                 | 7.95            | 1.00       | 7.99  | 3         | 7.90             | .97        | 8.27  | 2.5       |
| Purkof                  | 7.76            | 1.03       | 7.56  | 2.5       | 7.72             | 1.02       | 7.74  | 2         |

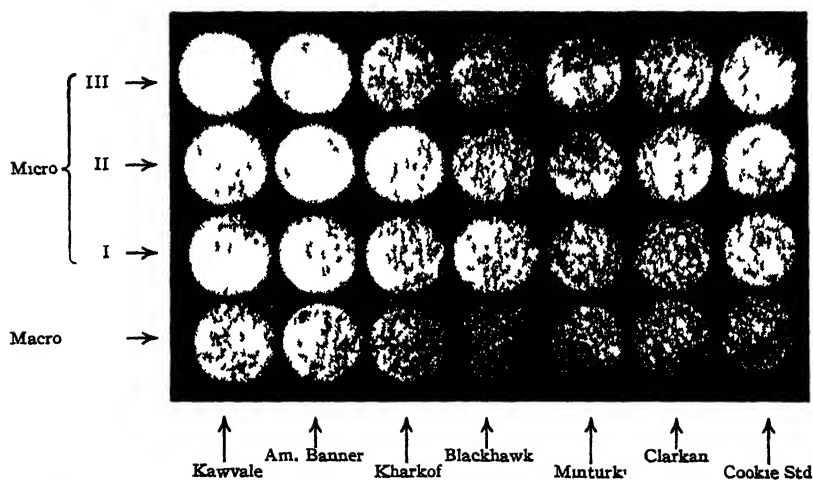


Fig. 2a Top grains and relative diameters for cookies baked from six pure varieties and a composite cookie standard by a macro and 3 micro procedures

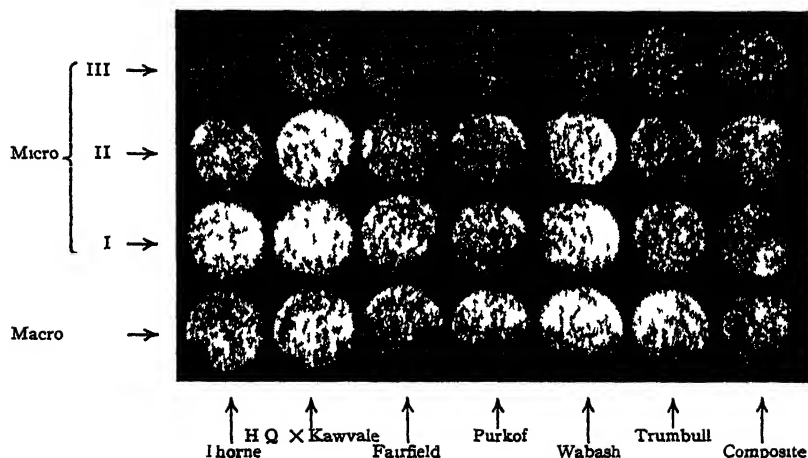


Fig. 2b Top grains and relative diameters for cookies baked from six pure varieties and a composite by a macro and three micro procedures

and sodium bicarbonate. A 38 g. weighing of this creamed mixture was creamed for three minutes with 9 to 10.5 ml. of water in which the .3 g. of ammonium bicarbonate was dissolved. The remaining procedures were carried out as described for Micro Method I.

*Micro Method III.* The third micro method differed from the second in the manner of adding the ammonium bicarbonate and sodium chloride. Since small quantities of ammonium bicarbonate affect cookie spreading (2), and ammonium bicarbonate in the form of dry salt or in solution decomposes appreciably at room temperature,

the ammonium and bicarbonate ions were added as two separate solutions. Solution A contained 127.68 g. sodium bicarbonate in 2000 ml. Solution B contained 135.53 g. ammonium chloride and 118.47 g. sodium chloride in 2000 ml. Five ml. solution A plus 3 ml. solution B furnished .3 g. ammonium bicarbonate and .4 g. sodium chloride.

Sugar 900 g., shortening 450 g., sodium bicarbonate 15 g. and non-fat milk solids 45 g. were creamed together in a Hobart mixer as de-

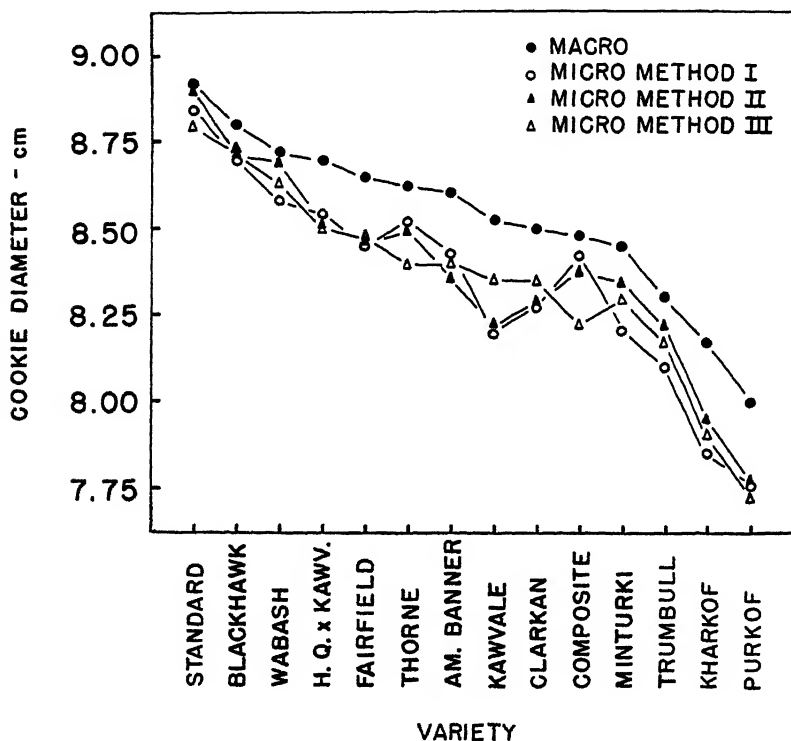


Fig. 3. Relative ranking according to cookie diameter of 12 pure varieties and two composites by a macro and three micro procedures.

scribed for Micro Method I. A 37.6 g. portion of this creamed mixture was creamed with 5 ml. solution A, 3 ml. solution B, and .8 to 2.3 ml. water in the micro mixer for three minutes at 178 rpm. Solutions A and B were dispensed from automatic pipettes and the water from a burette. The remaining procedures were as described for Micro Method I.

### Results

Cookie diameter (D), thickness (T), and spread factor (D/T) obtained for each of the 14 flours by the four cookie baking methods are summarized in Table III.

Representative cookies are reproduced photographically in Fig. 2 (a and b). The relative ranking of the 12 pure varieties and two composites by the four methods according to diameter are shown graphically in Fig. 3. Diameter instead of  $D/T$  was plotted against variety because data obtained in the laboratory indicated a comparatively large variability in thickness measurements for replicate cookie bakes which checked closely in diameter. Since  $D/T$  values are a function of thickness as well as diameter, it was believed that a more accurate and reproducible characterization of cookies could be obtained by using diameter alone.

The data show that the micro methods agree closely with the macro procedure in ranking the varieties. Micro Method III, now used in routine baking by the Federal Soft Wheat Laboratory, appears to agree somewhat more closely with the macro method than the other two micro methods in ranking the varieties. It may be noted that although the cookies baked by the macro method generally spread somewhat more than those baked by micro procedures, the latter nevertheless showed a greater range in cookie diameters.

These results indicate that there is considerable flexibility in the way in which cookie doughs may be mixed without appreciably affecting either the rank of or spread between varieties. Furthermore, it appears to make little difference in variety evaluation work whether the flour is mixed in at low speed with a cake paddle or at high speed with pins in the planetaries and bowl, as for bread doughs, as long as the total amount of mixing action is kept at a minimum.

#### Acknowledgment

Credit is due Charles E. Bode, Federal Soft Wheat Laboratory, Wooster, Ohio, for assistance in certain phases of the cookie baking.

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## THE NUTRITIONAL VALUE OF WHITE BREAD CONTAINING NONVIALE DRIED YEAST<sup>1</sup>

R. D. SEELEY, H. F. ZIEGLER, JR., and R. J. SUMNER<sup>2</sup>

### ABSTRACT

The nutritive value of white breads containing 1 to 3% dried, non-viable, debittered, brewers' yeast and nonfat milk solids was studied in feeding tests for growth on weanling rats. Baked bread was the only ingredient in the diets except for a fat-soluble vitamin supplement. Biological values were determined for some of the breads at the 10% protein level in rations supplemented with a salt and vitamin mixture. The feeding to weanling rats of white breads containing 1 and 3% dried yeast produced, respectively, increases in the daily weight gain of 0.34 g. and 0.66 g. above the gain produced with basic formula white bread. The weight gain of the rats receiving the white bread without dried yeast was 0.55 g. per day. The food utilization of the breads baked with 1 and 3% dried yeast was increased from 9.2 g. gain per 100 g. food intake for white bread without yeast to 12.3 and 14.7 g. gain, respectively. Similar increases were observed when white breads containing 1 and 3% nonfat milk solids plus dried yeast were fed to growing rats. Under the experimental conditions employed, dried yeast was a better supplement than nonfat milk solids since breads containing either 1% dried yeast or 3% nonfat milk solids were approximately equal in promoting growth of weanling rats when fed as the only source of food. However, the largest weight gains were observed when the bread contained both nonfat milk solids and dried yeast.

The inclusion of dried yeast in white bread did not produce any marked increase in the biological value of the bread proteins as measured by a nitrogen balance method. The small increases in the protein content of the breads containing dried yeast, and perhaps known or unknown members of the B complex in the yeast, appear to play a role in producing the increased growth response in rats.

The relatively low nutritive value of patent white flour or bread made with patent white flour as compared to whole wheat flour has been demonstrated by many investigators (5, 10, 15, 16) and has been attributed primarily to a loss in vitamins and to a lower biological value of the protein in the flour. Riggs *et al.* (17) observed that the "enrichment" of white flour in bread causes only a slight improvement in the growth response of rats. Westerman and Hall (23) found that the addition of calcium pantothenate and pyridoxine increases the growth of rats on an "enriched" white flour diet. Mitchell and Block (14) state that the limiting essential amino acid of white flour is

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lysine. Valine has also been reported as a limiting essential amino acid (12). Light and Frey (12) report that the supplementary value of nonfat milk solids and nonviable dried yeast to white bread is primarily due to this lysine content. Sure (19, 20, 21), in an extensive series of studies on the supplementation of cereal grains, observed that rats fed "enriched" white flour supplemented with 1 to 5% nonviable dried yeast exhibited large increases in weight gain and food utilization as compared to those fed unsupplemented "enriched" white flour. These increases were not entirely accounted for by the lysine content of the yeast. The diets employed by Sure contained adequate amounts of eight crystalline B-vitamins. However, the supplementary value of dried yeast for white flour was not determined in the presence of nonfat milk solids or in baked bread.

The following study was undertaken to determine the nutritive value of white breads containing 1 and 3% nonviable, dried, debittered brewers' yeast, as measured by rat growth when the breads were the sole ingredient in the diet except for a fat-soluble vitamin supplement. As most bread in this country is baked with nonfat milk solids, the growth response of rats fed bread containing dried yeast was studied in the presence and absence of this ingredient. Biological values measured by the nitrogen balance method in growing rats were conducted to determine the effect of these ingredients on the utilization of the protein of white bread.

### Materials and Methods

The bread used in these experiments was made by the sponge dough process, using 72% extraction flour according to the basic bread formula given in Table I. The dried yeast and nonfat milk solids<sup>3</sup>

TABLE I  
BASIC BREAD FORMULA

| Ingredient               | Parts |
|--------------------------|-------|
| Flour                    | 100.0 |
| Water                    | 62.0  |
| Sugar                    | 6.0   |
| Salt                     | 2.0   |
| Hydrogenated Shortening  | 3.0   |
| Baker's Compressed Yeast | 2.0   |
| Mineral Yeast Food       | 0.5   |
| Diastatic Dri-Malt       | 0.5   |

were added at the dough stage, the percentages shown being based on the flour content. The dried yeast employed in these studies was a nonviable, debittered, brewers' yeast.<sup>4</sup> The bread was sliced, air-

<sup>3</sup> Spray-dried, nonfat milk solids from the Pevely Dairy Co., St. Louis, Missouri.

<sup>4</sup> Strain K, Anheuser-Busch, Inc., St. Louis, Missouri.

dried, and ground before being fed to the animals. Groups of eight weanling rats (4 males and 4 females) weighing between 45 and 55 g. were fed the various breads as the only ingredient in the diets, *ad libitum*, except for a fat-soluble vitamin supplement, for a 10-week growth period. Each rat received 800 units of vitamin A, 160 units of vitamin D, and 6 mg. of alpha-tocopherol per week. Weight gains and food consumption were recorded weekly.

Biological values were determined at the 10% protein level on some of the breads fed to growing rats, using the nitrogen balance technique of Mitchell (13). The diets and vitamin supplements used are recorded in Table II. Nitrogen content of the urine and feces was determined by a micro-Kjeldahl method.

The breads were analyzed for thiamin by the method of Hennessy and Cerecedo (8), for riboflavin by the method of Tobin and Rehm (22),

TABLE II  
BREAD DIETS FOR BIOLOGICAL VALUE DETERMINATIONS \*

|                     |       |
|---------------------|-------|
| White Bread (Dried) | 81.2  |
| Sucrose             | 3.8   |
| Salt Mixture **     | 4.0   |
| Cellu Flour         | 2.0   |
| Crisco              | 9.0   |
|                     | 100.0 |

\* The following crystalline vitamins were added per 1 000 g. of diet: 5 mg. thiamin hydrochloride, 10 mg. riboflavin, 5 mg. pyridoxine hydrochloride, 50 mg. calcium pantothenate, 20 mg. niacin, 1 000 mg. choline chloride, 400 mg. inositol and 50 mg. p-amino benzoic acid.

\*\* Wesson's modified Osborne Mendel salt mixture.

for niacin by the microbiological technique of Landy and Dicken (11), for pantothenic acid by the method of Strong, Feeney and Earle (18), and for pyridoxine by the procedure of Atkin, Schultz, Williams, and Frey (1). Total nitrogen, ash, moisture, calcium and phosphorus were also determined on the air-dried bread.

## Results

The growth response of weanling rats fed "enriched" white bread and breads baked with and without 1 and 3% nonviable, debittered, brewers' yeast and/or 1 and 3% nonfat milk solids is shown in Fig. 1. Over a ten-week period the rats on basic-formula white bread (curve with open squares) gained only 40 grams. Small increases in growth response were produced by "enriched" <sup>5</sup> white bread and the addition of 1% milk solids. One per cent dried yeast in white bread produced a weight gain of 68 g. over ten weeks, which was slightly greater than the weight gain of rats fed bread containing 3% of nonfat milk solids.

<sup>5</sup> Enriched with thiamin, riboflavin, niacin, and iron in accordance with flour standards (6).

Bread containing 3% dried yeast caused a weight gain of 92 g. over a ten-week period. The addition of dried yeast to white breads containing 1 and 3% of nonfat milk solids produced significantly greater weight gains in rats than bread containing only nonfat milk solids. The greatest growth (110 g.) was produced by bread containing 3% nonfat milk solids and 3% nonviable, debittered brewers' yeast (curve with open circles).

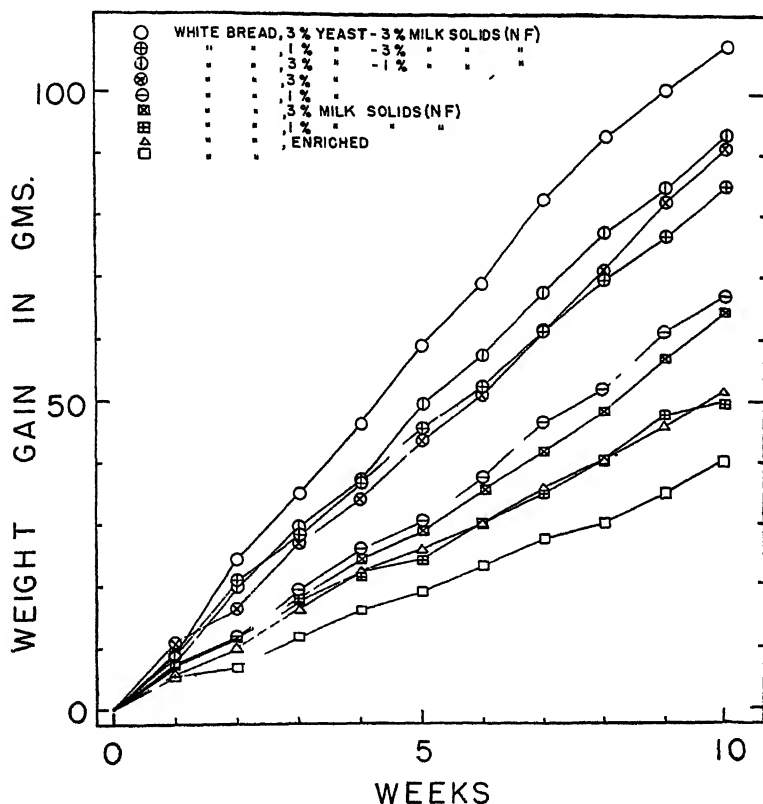


FIG. 1. The growth response of weanling rats fed, *ad libitum*, various breads.

The feeding of white breads containing nonfat milk solids and dried yeast results not only in increased weight gains as compared to those obtained with basic-formula white bread, but also in increased food consumption and food utilization. The food intakes, weight gains and food utilizations are recorded in Tables III and IV. These data were calculated for the usual six-week period. The food utilization of the various breads was not significantly different after a ten-week period.

TABLE III  
THE FOOD CONSUMPTION, WEIGHT GAIN AND FOOD UTILIZATION  
OF VARIOUS BREADS FED TO RATS

| Type of bread                     | Food intake<br>g /day | Gain in<br>body<br>weight<br>g /day | Increase <sup>1</sup><br>in body<br>weight<br>% | Food utilization                    |                            |
|-----------------------------------|-----------------------|-------------------------------------|-------------------------------------------------|-------------------------------------|----------------------------|
|                                   |                       |                                     |                                                 | G gain per<br>100 g bread<br>intake | Increase <sup>1</sup><br>% |
| White Bread                       | 5.99                  | 0.55                                | —                                               | 9.2±0.6**                           | —                          |
| White Bread "Enriched"            | 6.56                  | 0.72                                | 31                                              | 11.0±0.7                            | 20                         |
| White Bread+1% Nonfat Milk Solids | 6.40                  | 0.72                                | 31                                              | 11.2±0.7                            | 22                         |
| White Bread+3% Nonfat Milk Solids | 6.79                  | 0.84                                | 53                                              | 12.4±0.6                            | 35                         |
| White Bread+1% Dried Yeast        | 7.28                  | 0.89                                | 62                                              | 12.2±0.5                            | 33                         |
| White Bread+3% Dried Yeast        | 8.22                  | 1.21                                | 120                                             | 14.7±0.8                            | 60                         |

\* Compared to Unsupplemented White Bread

\*\* Mean deviation of the mean =  $e = \sqrt{\frac{\sum d^2}{n(n-1)}}$

The food consumption of the basic-formula white bread was 5.99 g. per day, which resulted in a weight gain of 0.55 g. per day and a food utilization of 9.2 g. gain per 100 g. food intake. The rats fed "enriched" bread exhibited a daily weight gain of 0.72 g. and a food utilization of 11.0 g. White bread containing 1% nonfat milk solids produced the same results. The weight gain and food utilization of rats fed white bread containing 3% nonfat milk solids and white bread baked with 1% dried yeast were approximately equal. The weight gain and food utilization of rats fed white bread with 1% dried yeast were increased 0.34 and 3.0 g. per day, respectively, above the results with basic-formula white bread. The protein contents of the bread containing 3% nonfat milk solids and of the bread containing 1% dried

TABLE IV  
THE FOOD CONSUMPTION, WEIGHT GAIN, AND FOOD UTILIZATION  
OF VARIOUS BREADS FED TO RATS

| Type of bread                                       | Food intake<br>g /day | Gain in<br>body<br>weight<br>g /day | Increase <sup>1</sup><br>in body<br>weight<br>% | Food utilization                    |                            |
|-----------------------------------------------------|-----------------------|-------------------------------------|-------------------------------------------------|-------------------------------------|----------------------------|
|                                                     |                       |                                     |                                                 | G gain per<br>100 g bread<br>intake | Increase <sup>1</sup><br>% |
| White Bread+3% Nonfat Milk Solids                   | 6.79                  | 0.84                                | —                                               | 12.4±0.6**                          | —                          |
| White Bread+3% Nonfat Milk Solids<br>1% Dried Yeast | 8.14                  | 1.24                                | 48                                              | 15.2±0.2                            | 23                         |
| White Bread+3% Nonfat Milk Solids<br>3% Dried Yeast | 8.70                  | 1.64                                | 95                                              | 18.9±0.4                            | 52                         |
| White Bread+1% Nonfat Milk Solids<br>3% Dried Yeast | 8.10                  | 1.36                                | 62                                              | 16.8±0.5                            | 35                         |

\* Compared to 3% Nonfat Milk Solids Bread.

\*\* Mean deviation of the mean =  $e = \sqrt{\frac{\sum d^2}{n(n-1)}}$

yeast (Table VI) were almost identical (12.7 and 12.8%, respectively). The bread containing 3% dried yeast produced a still larger weight gain (1.21 g./day) and food utilization (14.7 g. gain per 100 g. of food intake). These data demonstrate that when white bread is fed, *ad libitum*, as the sole food source to the weanling rat on an equal percentage basis, the addition of dried yeast is more efficient for growth than nonfat milk solids.

As most commercial bread in the United States contains milk solids, the value of adding dried yeast to such bread was determined. The inclusion of 3% nonfat milk solids in bread is a "representative" figure for this ingredient in commercial bread baking. These data are recorded in Table IV. The addition of 3% nonviable, debittered, dried brewers' yeast to bread containing 3% of nonfat milk solids produced a significant increase in food consumption of 1.91 g. per day and an increase in weight gain of 0.80 g. per day. Food utilization

TABLE V  
BIOLOGICAL VALUES AND DIGESTIBILITIES OF VARIOUS BREADS

| Type of bread                       | Biological value | Digestibility           |
|-------------------------------------|------------------|-------------------------|
| White Bread                         | 51.7 ± 1.3*      | 92.0 ± 0.2 <sup>1</sup> |
| White Bread + 3% Nonfat Milk Solids | 54.1 ± 1.5       | 91.9 ± 0.7              |
| White Bread + 3% Dried Yeast        | 51.8 ± 1.5       | 90.6 ± 0.5              |
| White Bread + 3% Nonfat Milk Solids |                  |                         |
| 1% Dried Yeast                      | 51.6 ± 1.5       | 89.5 ± 0.5              |
| White Bread + 3% Nonfat Milk Solids |                  |                         |
| 3% Dried Yeast                      | 52.3 ± 1.1       | 88.1 ± 0.5              |
| White Bread + 1% Nonfat Milk Solids |                  |                         |
| 3% Dried Yeast                      | 51.7 ± 2.0       | 91.2 ± 0.9              |
| Lactalbumin                         | 84.2 ± 1.1       | 97.4 ± 0.5              |

\* Mean deviation of the mean  $= \epsilon = \sqrt{\frac{\sum d^2}{n(n-1)}}$

was raised to the extent of 6.5 g. gain per 100 g. of food intake. Similar increases in nutritive value were produced with the other combinations of dried yeast and nonfat milk solids in white bread.

The value of dried yeast as an additive to bread has been attributed largely to its lysine content. Biological values of some of the breads used in the growth studies were determined in growing rats, using the nitrogen balance method. The rations were compounded so as to contain 10% protein supplied by the bread. The results are given in Table V. Although the biological values and digestibility are discussed as protein utilization, the nitrogen balance method actually measures nitrogen utilization which is assumed to be related directly to the protein utilization.

The addition of 3% of nonfat milk solids and 3% of dried yeast

either alone or in combination did not produce any marked increase in the biological value of the proteins of the baked bread. The largest biological value of 54.1% for bread containing 3% of nonfat milk solids was not significantly greater ( $P = 0.25$ )<sup>6</sup> than that for the white bread. For the number of animals used in these determinations, an increase of 4 in biological value would have to be obtained to be statistically significant ( $P = 0.05$ ). Larger numbers of animals would be required to determine smaller differences in biological values of these breads. The average biological value of 51.7% for white bread made with flour of 72% extraction compares well with the value of

TABLE VI  
CHEMICAL COMPOSITION OF BREADS

| Type of bread                               | Moisture % | Total protein % | Calcium % | Phosphorus % | Ash % | Thiamine $\gamma/\text{g.}$ | Riboflavin $\gamma/\text{g.}$ | Niacin $\gamma/\text{g.}$ | Pantothenic acid $\gamma/\text{g.}$ | Pyridoxine $\gamma/\text{g.}$ |
|---------------------------------------------|------------|-----------------|-----------|--------------|-------|-----------------------------|-------------------------------|---------------------------|-------------------------------------|-------------------------------|
| White Bread                                 | 7.91       | 12.0            | 0.10      | 0.12         | 2.55  | 0.9                         | 1.1                           | 12.1                      | 5.2                                 | 0.6                           |
| White Bread "Enriched"                      | 7.91       | 12.3            | 0.10      | 0.12         | 2.54  | 3.9                         | 2.6                           | 35.8                      | 5.2                                 | 1.1                           |
| White Bread +1% Milk Solids*                | 7.78       | 12.6            | 0.14      | 0.13         | 2.61  | 1.1                         | 1.1                           | 12.7                      | 5.5                                 | 1.0                           |
| White Bread +3% Milk Solids*                | 7.81       | 12.7            | 0.15      | 0.14         | 2.72  | 1.1                         | 1.5                           | 11.0                      | 4.6                                 | 1.1                           |
| White Bread +1% Dried Yeast                 | 7.90       | 12.8            | 0.10      | 0.14         | 2.61  | 2.2                         | 1.1                           | 19.9                      | 6.3                                 | 1.2                           |
| White Bread +3% Dried Yeast                 | 8.01       | 13.4            | 0.09      | 0.17         | 2.69  | 4.1                         | 2.2                           | 25.6                      | 7.0                                 | 2.1                           |
| White Bread +3% Milk Solids* 1% Dried Yeast | 8.43       | 13.3            | 0.17      | 0.16         | 2.68  | 2.0                         | 1.3                           | 19.8                      | 5.5                                 | 0.9                           |
| White Bread +3% Milk Solids* 3% Dried Yeast | 8.03       | 13.9            | 0.18      | 0.18         | 2.82  | 3.5                         | 2.4                           | 22.7                      | 6.5                                 | 2.1                           |
| White Bread +1% Milk Solids* 3% Dried Yeast | 7.93       | 13.8            | 0.12      | 0.17         | 2.54  | 3.9                         | 1.7                           | 26.4                      | 6.4                                 | 1.6                           |

\* Nonfat Milk Solids.

51.2% reported by Henry and Kon (4) for white bread made with flour of 70% extraction.

The digestibility of the protein of the white bread was decreased slightly by the addition of 3% dried yeast. The lowest digestibility of the protein of the breads studied was 88.1% for white bread containing 3% each of dried yeast and nonfat milk solids. The digestibility of the protein of the white bread made by the basic formula was 92.0%.

The increase in food utilization observed in the growing rats with dried yeast does not seem to be largely the result of amino acid supple-

<sup>6</sup> Calculated by Fisher "t" values (7).

mentation. As these determinations were conducted on diets of equal protein content containing eight crystalline B-vitamins plus 2% of a salt mixture, the biological values represent protein utilization *per se*. It may be possible that when the breads are fed as the sole food source in the diet of the growing rat, the B-vitamins and other essential substances in the ration may not produce the same protein utilization as they do when measured under the conditions of the biological value determination.

The chemical analyses of the breads for moisture, protein, mineral elements, and B-vitamins, as air-dried for the nutritional studies, are recorded in Table VI. The addition of nonfat milk solids and dried yeast produced increases in the protein content of about 1 to 2% on the air-dry basis of approximately 8% moisture. The addition of nonfat milk solids increased the calcium content, as would be expected. Yeast did not affect the calcium content, but produced small increases in the amount of phosphorus.

Except for a slight increase of thiamin in the 3% dried yeast bread, the levels of thiamin, riboflavin, and niacin in the breads containing dried yeast or nonfat milk solids did not exceed "enriched" white bread. The addition of dried yeast to the white bread produced small increases in the levels of pyridoxine and pantothenic acid. The growth response of the rats on white bread containing dried yeast cannot be attributed entirely to the small increase in the content of these two vitamins, as Sure (20) has reported that the addition of dried yeast to "enriched" white flour markedly improved the nutritive value in the presence of a supplement of eight synthetic B-vitamins in the diet.

### Discussion

The inclusion of nonviable, debittered, dried brewers' yeast in white bread increased the nutritive value markedly, even in the presence of 1 and 3% of nonfat milk solids, as measured by rat growth. The increased growth response may have resulted from (a) an increase in the biological value of the bread proteins, (b) an increase in the mineral content, (c) an increase in the vitamin content, (d) the higher protein content of the bread.

Sure (21) reported that the increase in protein efficiency of "enriched" white flour supplemented with 3 to 5% of dried yeast was greater than could be obtained with a quantity of lysine equal to the dried yeast in a diet containing a supplement of eight crystalline B vitamins. Sure did not add valine to his diets. In our determinations of the biological value of the various breads, the addition of either dried yeast or nonfat milk solids at the 3% level or lower did not



produce any significant increases in biological value. Henry *et al.* (9) in an excellent study on the biological value of white bread using the nitrogen balance method, observed that "the increase in biological value of the nitrogen of white bread caused by the addition of milk was slight." These authors further state that at these low levels of admixture (2 and 6% milk solids) there is no supplementary relationship between the milk and bread proteins. Although dried yeast contains 3.5% lysine (21) and is, therefore, an excellent source of this substance, the amounts of lysine and valine which it provides at the low level employed evidently were not enough to produce a large increase in the biological value of the bread proteins.

An important mineral deficiency of white bread is calcium (9). The improved growth response obtained with the addition of nonfat milk solids may be partially the result of the calcium which it provides as reported by Henry *et al.* (9). In the case of white bread containing dried yeast there is no increase in calcium content of the bread. The possibility of other minerals from dried yeast having an effect on growth will require further investigation with various mineral mixtures included in the rat diets.

The bread made with dried yeast did not contain larger amounts of thiamin, riboflavin or niacin than the "enriched" bread. Westerman and Hall (23) have observed that the addition of calcium pantothenate and pyridoxine improved the growth of rats on a B-complex-free, 20% casein diet plus "enriched" flour. However, Sure (20) observed that rat growth was improved by the inclusion of 1 to 3% dried yeast even in a ration containing an adequate supplement of 8 crystalline B vitamins. Cannon (4), using the rat repletion technique, observed that the addition of 3% dried yeast to a bread containing 2% of nonfat milk solids fed in a ration containing 6 crystalline B vitamins and a liver concentrate increased the nutritive value 20 to 33%, practically equalling whole wheat bread. Part of the increase in the growth response of rats fed white bread containing dried yeast under the conditions of the experiments employed is the result of an increased B vitamin content. However, as observed by Sure and by Cannon, the growth response may occur even in the presence of known B-complex supplements.

The increased protein content of the breads containing dried yeast and nonfat milk solids undoubtedly is a factor in improving growth response of rats fed the various baked breads. The fraction of ingested protein utilized by the rat for maintenance declines as the protein intake increases (2). Thus, rats receiving breads of higher protein content or rats with an increased food consumption will have a greater fraction of protein available for growth. Food utilization will be

increased in a similar manner. Mitchell observed that when paired-feeding experiments were conducted with rats, the difference between the nutritive values of "enriched" bread and bread containing 6% nonfat milk solids was less than with *ad libitum* feeding, although the superiority of the milk bread was still evident. The protein contents of the diets were not equalized in these experiments.

The increased growth response of rats fed white bread containing dried yeast is not primarily due to a large increase in the biological value, but appears to be the result of a higher vitamin and protein content which increases the food and protein consumption. Further experiments are in progress to determine the exact nature of the factors which produce the larger growth responses when white bread is supplemented with dried yeast. The white bread which contained both dried yeast and nonfat milk solids at the 3% level had the highest nutritive value, as measured by rat growth, in these studies.

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## PREPARATION OF WHEAT FLOUR PENTOSANS FOR USE IN RECONSTITUTED DOUGHS<sup>1</sup>

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### ABSTRACT

The separation of wheat-flour pentosans of satisfactorily low nitrogen content for baking tests was accomplished either by selective adsorption of the proteins of a heated, centrifuged flour extract on a suitable agent, or by precipitation of the proteins with the barium hydroxide-zinc sulfate reagent of Somogyi; dialysis then removed most of the remaining nitrogenous and carbohydrate impurities. The extracts were heated to destroy pentosanase activity. Yields were about 8 and 5 g. of pentosans per kg. of flour with the adsorption and the precipitation method respectively. The precipitation method gave a product of higher purity and of lower average molecular weight, although pentosans obtained with either method showed the chemical characteristics noted by earlier workers, particularly gel formation upon oxidation.

Under the conditions of this study, soluble pentosans had little effect on the baking performance of doughs reconstituted without the water-soluble fraction of flour, but the handling properties of the doughs themselves were distinctly modified. The pentosans corrected to a large degree the slack softness, wet surface, and lack of normal stickiness shown by doughs reconstituted with only gluten and starch. Improvements in volume, grain and texture were small.

The importance of water-soluble components in the bread-baking performance of flours from some wheat varieties was demonstrated by Finney (6), who separated the glutens, starches, and water-soluble fractions of flours and recombined them in the original proportion and

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in different proportions before baking them into bread. Among constituents known to be present in the water-soluble fraction of flour, the pentosans have been studied in some detail by Baker, Parker, and Mize (2); the observed formation of an irreversible gel upon treatment of pentosan solutions with certain oxidizing agents used as bread improvers suggested to these workers that soluble pentosans may have an important influence on dough properties. It seemed desirable, therefore, to determine more clearly the actual contribution of pentosans to the baking behavior of a flour. To facilitate such studies, improved methods were developed for the preparation of pentosans in sufficient quantity for reconstitution baking.

### Methods and Materials

Pentosans were determined routinely by the colorimetric method of Brown (3), which gave results in satisfactory agreement with those obtained by a furfural distillation method (1). The baking formula of Finney and Barmore (7), with optimal bromate, was used in conjunction with the A.A.C.C. Standard Baking Procedure (1), and mixing times were determined with the National Recording Mixograph.<sup>3</sup> Doughs were reconstituted with wet gluten by the method of Sandstedt, Jolitz, and Blish (10), and the water-soluble fractions were prepared according to the recommendations of Finney (6). A single uniform lot of starch was used for all doughs. Glutens were stored in the frozen state, and water-soluble preparations were dried by lyophilization. The flours used were straight-grade, unbleached flours experimentally milled from samples of pure varieties. One sample contained 13.1% protein (at 14% moisture) and was milled from Turkey wheat grown in Texas; the other was an 8.6% protein flour from Purkof, a semi-hard red winter wheat, grown in Indiana.

*Preparation of Pentosans.* Various types of adsorbing agents were investigated for their selectivity toward the nitrogen-containing materials of flour extracts in an effort to separate the pentosans and proteins by a method which would give good yields of pentosans and allow recovery of proteins by elution. The agents used included Norite A, kaolin, bentonite, permutit, synthetic resins of the Amberlite type, and the activated clays marketed by the Filtrol Corporation.<sup>3</sup> Of these, Special Filtrol was found to be the most satisfactory from the standpoint of efficiency of protein adsorption, ease of handling, and inertness toward pentosans. Up to 90% of the non-dialyzable nitrogen of a flour extract could be removed by this agent, while the pentosan content was scarcely affected. However, no effective method of recovery of the adsorbed protein has been devised.

<sup>3</sup> Mention of trade names of material and equipment does not constitute endorsement by the Department of Agriculture.

It was necessary to heat flour extracts to 90° to 95°C. for 3 to 4 minutes to prevent a rapid decrease in viscosity of the extracts. Although the heating caused precipitation of 30 to 50% of the total nitrogen of the extracts, the viscosity remained virtually unchanged. These observations suggested that pentosans were the main contributor to the viscosity of the extracts and that enzymatic action upon the pentosans was responsible for the viscosity changes observed. Baker, Parker, and Mize (2) had observed evidence of pentosanase activity in bran extracts, and Dr. Parker<sup>4</sup> informed us that subsequent work had shown the presence of a pentosanase in flour. Accordingly, the identity of the enzyme in the flour extracts was established by the viscosity-decreasing effects of the extracts upon solutions of purified pentosans. The adsorption agents failed to remove the pentosanase completely from solution.

Another method of preparation of pentosans was developed from an investigation of the effect of protein-precipitating agents on the solubility of pentosans. Treatment of a flour extract with the barium hydroxide-zinc sulfate reagent of Somogyi (11) produced sparkling clear filtrates containing practically none of the original non-dialyzable nitrogen and about 60% of the original pentosans of the extract. This reagent also lowered the pentosanase activity of unheated extracts to almost insignificant levels.

A more detailed description of the two methods for the isolation of soluble flour pentosans follows. Extracts were prepared by stirring a quantity of flour with four parts of the 0.1%, pH 6.8, phosphate buffer of Dill and Alsberg (5) for 30 minutes. After removal of the solids by centrifugation, the extract was heated to 90° to 95°C. for 3 to 4 minutes and cooled immediately. The precipitated material was removed by centrifugation. In the adsorption method, the supernatant was shaken or stirred for 30 minutes with five grams of Special Filtrol per 100 ml. of solution. For best results the pH for this step should be in the range of 5.5 to 6.0. Actually, the pH usually was found in this range after the addition of Filtrol to the extract. The Filtrol was then removed by centrifugation, and the supernatant dialyzed in Visking<sup>3</sup> cellulose tubing against distilled water, concentrated under vacuum below 40°C., and lyophilized.

In the second method, one volume each of 0.3 *N* barium hydroxide and 5% zinc sulfate were added successively, with stirring, to 3 volumes of the supernatant after heat treatment. The resulting solution should be neutral or only very slightly alkaline. The heavy, white precipitate was removed by filtration, and the filtrate was dialyzed, concentrated, and lyophilized as described above.

<sup>4</sup>Personal communication.

**Characterization of Pentosans.** Typical preparations of pentosans obtained by the adsorption method contained 70 to 80% pentosans and 3 to 5% protein without further purification. About 8 g. of pentosans were obtained per kg. of flour. Barium-zinc preparations contained up to 90% pentosans and 2 to 3% protein; about 5 g. were obtained per kg. of flour.

Preliminary ultracentrifuge examination of material prepared by the adsorption method showed two peaks of about equal area. The sedimentation constant of the sharper, more slowly moving boundary corresponded to a minimum molecular weight of about 15,000, disregarding corrections for asymmetry or hydration. A preparation by the barium-zinc method was single-boundaried without marked spreading, and therefore appeared to be a relatively homogeneous preparation. The observed sedimentation constant also corresponded to a minimum molecular weight of about 15,000. Osmotic pressure measurements by the method of Bull (4) on the same preparations

TABLE I

COMPARISON OF THE EFFECTS OF PENTOSANS AND COMPLETE WATER-SOLUBLE FRACTIONS ON LOAF VOLUMES OF RECONSTITUTED DOUGHS<sup>1</sup>

|                                              | Turkey              |                    | Purkof              |                    |
|----------------------------------------------|---------------------|--------------------|---------------------|--------------------|
|                                              | Average loaf volume | Standard deviation | Average loaf volume | Standard deviation |
| Control (gluten plus starch)                 | cc. 695             | cc. 13             | cc. 600             | cc. 19             |
| Control plus pentosans                       | 715                 | 14                 | 665                 | 14                 |
| Control plus complete water-soluble fraction | 835                 | 18                 | 800                 | 7                  |

<sup>1</sup> Gluten content of all doughs equivalent to that of 100 g. of flour at 12% protein. Pentosans and water-soluble fraction added in ratio to gluten as found in original flour.

gave average molecular weight values of approximately 22,000 for the barium-zinc preparation and approximately 39,000 for the Filtrol preparation. Apparently the barium-zinc reagent precipitated the larger pentosan particles as well as the proteins of a flour extract.

The pentosans obtained by these methods were slowly soluble in water to form clear, viscous solutions. A purplish red color was obtained with iodine. Oxidizing agents produced gels which did not liquefy on standing and which were little affected by vigorous stirring.

**Baking Studies.** Baking results obtained with reconstituted doughs prepared from the two flours are presented in Table I.

These values show that the complete water-soluble fractions produced volume responses of 140 and 200 cc. for the Turkey and Purkof doughs, respectively, while the volume increases for the

pentosans were only 20 and 65 cc. The slight effect of the pentosans can be partially accounted for by the small amounts of water-soluble protein remaining in the pentosan preparations. Neither the source nor method of preparation of the pentosans had an effect on the results obtained. Doughs without added pentosans were soft and slack, were not sticky, and had a wet, shiny surface. The doughs to which pentosans only had been added were firm and dry, and sticky to a normal degree. The grain and texture of loaves baked with the Purkof gluten were slightly improved by pentosans, but loaves with Turkey gluten were unaffected. A small decrease in absorption was noted for doughs from both flours in the absence of pentosans.

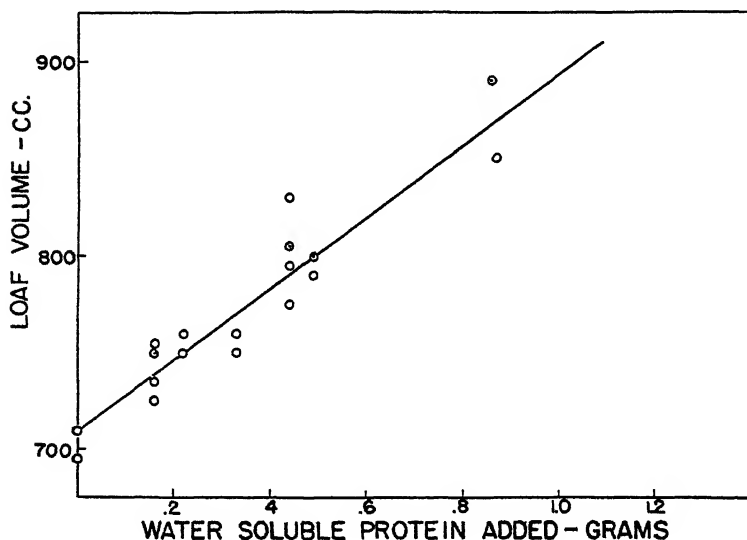


Fig. 1. Scatter diagram showing relation between loaf volume and amounts of protein in water-solubles added to reconstituted doughs. The original flour contained 0.87 g. water-soluble protein per 100 g. The gluten content of all doughs was equivalent to that of 100 g. of flour at 12% protein.

The small contribution of pentosans to loaf volume responses was also shown by use of a water-soluble fraction from which most of the proteins, but little of the pentosans and dialyzable constituents, had been removed by treatment with Special Filtrol. By the addition in varying amounts of treated or untreated fractions, reconstituted doughs were obtained which contained varied amounts and ratios of pentosans and water-soluble proteins. By this means a comparison of their relative effects on loaf volume was possible. The results obtained with Turkey flour fractions are presented in Figs. 1 and 2. Fig. 1 shows loaf volumes plotted against the amounts of protein contained in the added water-soluble material. The small dispersion

of points around the regression line emphasizes the highly significant correlation coefficient of  $+0.98$  which was obtained. A much lower order of correlation is obvious in Fig. 2, where the same loaf volume data are plotted against the amounts of pentosans contained in the added materials. The dispersion of points around the regression line is much greater, and the correlation coefficient is  $+0.77$ . The nitrogenous constituents, therefore, appear to be responsible for the loaf-volume-increasing effects of the water-soluble fraction. However, the baking experiments described do not exclude the possibility that water-soluble proteins may require the presence of pentosans to produce their optimum effect.

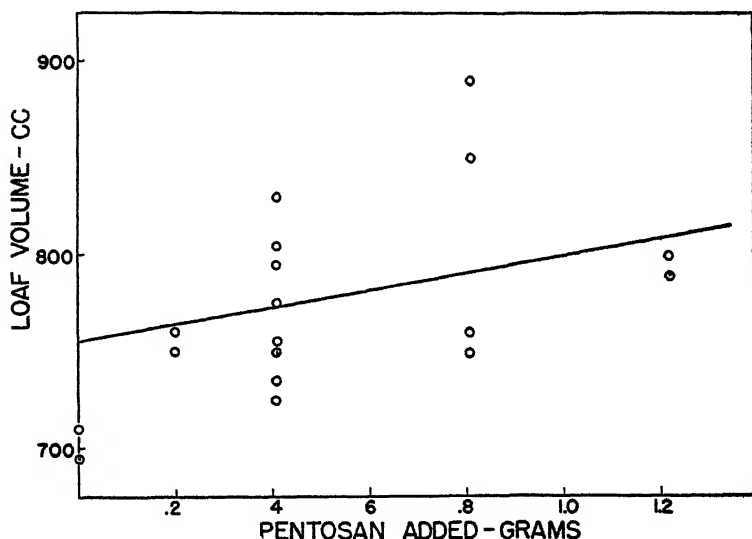


Fig. 2. Scatter diagram showing relation between loaf volume and amounts of pentosans in water-solubles added to reconstituted dough. The original flour contained 0.81 g. soluble pentosans per 100 g. The gluten content of all doughs was equivalent to that of 100 g. of flour at 12% protein.

### Discussion

The marked effect of temperature on the viscosity of pentosan solutions, described by Baker and co-workers (2), may explain the failure of pentosans to affect materially the behavior of doughs in the oven. These workers found that a solution which was very viscous at room temperature became almost water-thin after being warmed to  $65^{\circ}\text{C}$ . Thus, firmness imparted by pentosans would be drastically lessened as the temperature of a dough increased during baking.

The distinct modifying effects of soluble pentosans on the handling properties of reconstituted doughs were quite similar to those reported by Sandstedt, Jolitz, and Blish (10) for the small-granule fraction of



starch. Since Baker and co-workers (2) reported small-granule starch to contain up to 14% insoluble pentosans, it is possible that the effects of this starch fraction were due, in large part, to the insoluble pentosans. MacMasters and Hilbert (8) found only 4% pentosans in their preparations of small-granule starch, but it has been suggested<sup>5</sup> that pentosanase activity during preparation may have been partly responsible for this low value. Ofelt (9) obtained small-granule starch fractions containing approximately 8% pentosans but observed no effect of either this starch or the total water-soluble fraction on dough-handling properties. In this regard, Sandstedt and coworkers found that their small-granule starch fraction not only modified dough-handling properties but also decreased loaf volume and improved grain and texture. The divergency of all these results clearly indicates that a more extensive study is required to determine the importance of pentosans and small granule starch in the baking performance of flours of all types.

#### Acknowledgments

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<sup>5</sup> Personal communication from Dr. MacMasters.

# THE PROTEIN, NIACIN, AND PANTOTHENIC ACID CONTENTS OF CORN INBRED LINES<sup>1</sup>

LORRAINE D. RODRIGUEZ, CHARLES H. HUNT,  
and R. M. BETHKE<sup>2</sup>

## ABSTRACT

Thirty-nine corn inbred lines grown at Columbus, Ohio, in 1946 were analyzed for total protein, niacin, and pantothenic acid. The lines ranged from 9.9 to 17.8% protein (mean 13.1), from 12.4 to 54.1  $\mu\text{g}$ . niacin (mean 22.6), and from 2.6 to 9.4  $\mu\text{g}$ . pantothenic acid (mean 6.1). Tests for correlation coefficient  $r$  made on the assay results of the three components, each with the other, revealed no significant correlations. However, the  $r$  values for niacin and protein (negative) and for niacin and pantothenic acid (positive) approached the 5% level of significance, indicating that there may be some relationship between these components.

The chemical composition of the corn plant has been the subject of considerable study. Hybrids have been compared with open-pollinated varieties and attempts have been made to trace heritable characters in inbred lines and hybrids. Recently, attention has been given to vitamins, mineral elements, and amino acids of the corn grain, as well as to protein, oil, sugar, starch, and ash.

Experiment stations first produced high or low protein corn by seed selection (Hume, Champlin, and Loomis, 9; Smith, 14). Breeding high-protein corn by Mendelian methods was reported by Hayes (7).

Curtis and Earle (4) found variations attributable to heredity and/or environment in composition of grain, including protein, from 17 hybrids grown at different locations.

Sayre (13) reviewed the protein content of some open-pollinated varieties and hybrids tested at the Ohio Station during several years. Early studies showed that hybrids had a more uniform protein content than open-pollinated varieties. Open-pollinated corn grown with seven hybrids at several locations through four seasons did not always contain the lowest percent protein, but proved inferior to the hybrids in yield of protein per acre. He found that soil, season, and maturity affected protein content.

Hunt, Ditzler, and Bethke (10) have reported that while both heredity and environment affect niacin and pantothenic acid in

<sup>1</sup> Manuscript received August 31, 1949. Published with the approval of the Director of the Ohio Agricultural Experiment Station.

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hybrids, niacin is less subject to environmental influences than pantothenic acid. Further study showed the effect of specific inbred lines on both vitamins in various types of hybrids (Ditzler, Hunt, and Bethke, 5). After finding that inbred lines varied more widely than related hybrids in niacin and that hybrids high in niacin came from parents high in niacin, Gorfinkel (6) concluded that niacin in maize is a genetically inherited factor. Richey and Dawson (12) also reported wide variations in the niacin content of inbred lines. Hybrids were intermediate between the parents. These investigators believe that it would be possible to develop hybrids with 50  $\mu\text{g}$ . niacin per g., but that such hybrids would be valueless unless they possessed other desirable characteristics.

A few investigators have correlated vitamin content with other components of cereal grains. The work of Burkholder, McVeigh, and Moyer (2), Mather and Barton-Wright (11), and Cameron and Teas (3) indicates a correlation between relatively high niacin and sugary-type maize endosperm. Aurand and Miller (1) reported a definite influence of heredity on the carotene and crude protein contents of single cross corn hybrids but found no correlation between the two nutrients in the corn tested. Hoffer, Alcock, and Geddes (8) found a significant positive correlation ( $r = +0.326$ ) between thiamine and protein in 383 samples of hard red spring wheat. At this Station, Hunt and co-workers found a significant positive correlation ( $r = +0.834$ ) between niacin and protein in 28 oats samples grown during two seasons on a 5-year rotation fertility experiment (unpublished data).

The present study was undertaken to determine whether a correlation existed in corn inbred lines between niacin and pantothenic acid and between the contents of each of these vitamins and protein.

### Materials and Methods

The grain from thirty-nine corn inbred lines grown under open pollination at Columbus, Ohio, in 1946 was analyzed for total protein, niacin, and pantothenic acid. Total protein was determined by the Kjeldahl procedure. Niacin and pantothenic acid were determined microbiologically, using methods previously described (Hunt *et al.*, 1947). Results were calculated to dry matter basis. The test for correlation coefficient  $r$  was made on niacin with pantothenic acid and on protein with each of the two vitamins.

### Results and Discussion

The assay results are shown in Table I. The lines ranged from 9.9 to 17.8% protein (mean 13.1), from 12.4 to 54.1  $\mu\text{g}$ . niacin (mean

22.6), and from 2.6 to 9.4  $\mu$ g. pantothenic acid (mean 6.1) per g. sample. The ranges of all three components were quite wide. This result agrees with the findings of Gorfinkel (6) and Richey and Dawson (12) whose tests of inbred lines showed a wide range in niacin. When the three components were correlated with each other, the values of correlation coefficient  $r$  for these three components were

TABLE I

TOTAL PROTEIN, NIACIN, AND PANTOTHENIC ACID CONTENTS OF CORN  
INBRED LINES GROWN AT COLUMBUS, OHIO, IN 1946  
(Dry matter basis)

| Inbred line | Total protein<br>% | Niacin<br>mcg./g. | Pantothenic acid<br>mcg./g. |
|-------------|--------------------|-------------------|-----------------------------|
| Ind. WF9    | 12.1               | 25.8              | 8.2                         |
| CI.4-8      | 14.4               | 18.0              | 5.1                         |
| Ind. Tr     | 11.9               | 27.0              | 5.1                         |
| W23         | 9.9                | 33.0              | 6.7                         |
| CI.7        | 13.4               | 24.7              | 8.1                         |
| CI.187-2    | 12.7               | 18.2              | 5.8                         |
| H5          | 14.9               | 16.4              | 6.0                         |
| H7          | 17.8               | 27.9              | 5.9                         |
| Ia.1205     | 12.7               | 23.7              | 7.1                         |
| K4          | 14.4               | 21.4              | 9.0                         |
| K155        | 11.8               | 20.6              | 4.7                         |
| K166        | 12.8               | 24.0              | 7.6                         |
| Ia.L289     | 12.9               | 20.1              | 4.4                         |
| Ia.L317     | 13.8               | 12.4              | 4.1                         |
| III. M14    | 12.7               | 24.4              | 6.6                         |
| T8          | 12.3               | 19.1              | 6.1                         |
| Ia. Os 420  | 13.7               | 23.6              | 8.1                         |
| Ia. Os 426  | 13.4               | 18.7              | 4.2                         |
| III. A      | 14.7               | 23.7              | 5.8                         |
| III. Hy     | 11.1               | 15.8              | 3.3                         |
| Ind. 38-11  | 15.0               | 20.6              | 6.2                         |
| Ia. P8      | 13.2               | 17.8              | 5.8                         |
| Kys         | 15.7               | 25.8              | 7.2                         |
| Oh02        | 12.6               | 32.0              | 6.9                         |
| Oh04        | 13.0               | 24.4              | 6.4                         |
| Oh07        | 12.0               | 27.0              | 5.8                         |
| Oh7A        | 11.3               | 54.1              | 6.9                         |
| Oh26        | 13.6               | 15.8              | 6.6                         |
| Oh28        | 11.6               | 43.6              | 5.4                         |
| Oh33        | 12.6               | 13.3              | 4.6                         |
| Oh40B       | 12.9               | 13.6              | 7.3                         |
| Oh41        | 12.1               | 15.2              | 5.4                         |
| Oh51        | 11.0               | 16.8              | 6.2                         |
| Oh51A       | 11.3               | 23.0              | 5.5                         |
| Oh56        | 13.2               | 20.1              | 4.7                         |
| Oh56A       | 13.9               | 19.6              | 9.4                         |
| Oh65        | 14.6               | 13.2              | 2.6                         |
| Oh84        | 12.7               | 23.6              | 6.2                         |
| Oh93        | 14.0               | 22.4              | 6.3                         |
| Mean        | 13.1               | 22.6              | 6.1                         |
| Minimum     | 9.9                | 12.4              | 2.6                         |
| Maximum     | 17.8               | 54.1              | 9.4                         |

as follows: for protein with niacin,  $-0.249$ ; for protein with pantothenic acid,  $+0.086$ ; for niacin with pantothenic acid,  $+0.285$ . None of the values for  $r$  was significant for 39 pairs. However, the  $r$  values for protein with niacin and for niacin with pantothenic acid approach significance ( $r$  = approximately .31 at 5% level) and indicate that there is probably some relationship between the components compared.

#### Acknowledgment

The authors are indebted to J. D. Sayre, Department of Agronomy, Ohio Agricultural Experiment Station, in cooperation with the United States Department of Agriculture, for supplying the samples analyzed.

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## COMMUNICATIONS TO THE EDITOR

### The Lever System of the Baker Compressimeter <sup>1</sup>

SIR:

The Baker Compressimeter, since it was first reported by Platt,<sup>2</sup> has been used by a number of workers to follow the change in firmness or compressibility of bakery products during the progress of staling. Platt in his original paper stated, "When the plunger is depressed, the amount of the depression is multiplied 10 times and is read direct in millimeters on scale D." However, he did not mention any factor for converting force scale readings into actual depressing force.

Recently, Favor and Johnston<sup>3</sup> reported the use of the Baker instrument in a study of the effect of polyoxyethylene monostearate on the staling of bread. These authors stated that "The stress in grams read from the scale on the instrument was multiplied by 10 to show the actual stress applied by the lever system to the section of bread crumb, and to secure better correlation with similar readings taken from other types of compressibility testers."

Other workers<sup>4</sup> have made similar statements, but attempts to demonstrate a correlation between the Baker Compressimeter and the Universal Penetrometer in this laboratory indicated that some factor other than 10 was involved.

To clarify this matter all the levers on the Baker instrument, with the exception of the one bearing the stress indicator, were immobilized with Scotch tape. By hanging analytical weights at the point where force is normally applied, it was found that with only a slight error, due perhaps to lack of linearity in the spring, the force scale values in grams equalled the force applied at this point.

The Baker Compressimeter was dismantled and the various levers were measured carefully with a steel scale. The results are shown in Fig. 1.

From these measurements it was possible to validate Platt's statement that the amount of depression is read directly from the instrument scale. The lever system, HG/DG, produces a tenfold

<sup>1</sup> These observations were made incidental to the course of an investigation supported by the United States Department of Agriculture under the Research and Marketing Act, Contract No. A-IS-30909. The conclusions contained in this report are those of the authors and are not to be construed as necessarily reflecting the views or indorsement of the Department of Agriculture.

<sup>2</sup> Platt, W. and Powers, R. Compressibility of bread crumb. *Cereal Chem.* 17: 601-621 (1940).

<sup>3</sup> Favor, H. H., and Johnston, N. F. Effect of polyoxyethylene stearate on the crumb softness of bread. *Cereal Chem.* 24: 346-355 (1947).

<sup>4</sup> Noznick, P. F., and Geddes, W. F. Application of the Baker Compressimeter to cake studies. *Cereal Chem.* 20: 463-477 (1943).

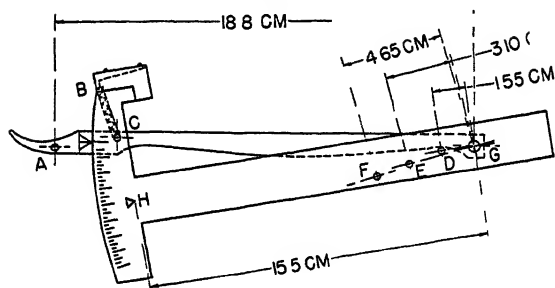


Fig. 1. Lever system of the Baker Compressimeter.

magnification of the depression, but the true depression is read in millimeters from the scale of the instrument. There is a small error resulting from angular displacement which may be ignored.

An examination of the levers involved in the force system revealed that for Plunger Position *D* the mechanical advantage is as follows:

$$\text{Mechanical Advantage} = \frac{AG}{CG} \times \frac{CG}{BG} \times \frac{BG}{DG} = \frac{AG}{DG}$$

From this observation and the lever measurements it was possible to calculate the theoretical conversion factor for the force scale for each of the three plunger positions. The following formula was employed:

$$f_A = f' \cdot \frac{d_A}{d'}$$

where:

$f_A$  = force applied to the sample by the plunger.

$f'$  = force applied to the lever at *A* and transferred to the plunger at *B* by a spring. This was determined to be the scale reading.

$d_A$  = length of the lever to which force is applied by a motor from the fulcrum, *G* to *A*.

$d'$  = length of the lever from *G* to the plunger where force is applied to the sample, *D*, *E* or *F*.

$\frac{d_A}{d'}$  = mechanical advantage of the system.

The results are as follows:

|             | Plunger Position |          |          |
|-------------|------------------|----------|----------|
|             | <i>D</i>         | <i>E</i> | <i>F</i> |
| $d_A$ , cm. | 18.8             | 18.8     | 18.8     |
| $d'$ , cm.  | 1.55             | 3.10     | 4.65     |
| Mech. Adv.  | 12.1             | 6.06     | 4.04     |

Having been assured that the Baker Compressimeter is a standardized machine<sup>5</sup> and that the one studied was a stock model, it would appear that the true factor to use in converting the force scale to actual force applied at the first plunger position (*D*) is 12.1 rather than 10.

By assuming that the lever-free Penetrometer provided accurate force-depression values on sponge rubber blocks, and by correlating these with Baker readings on the same blocks, it has been possible to demonstrate that the theoretical constants obtained are correct. The experimentally determined constants had an average deviation from the theoretical of 1.3% or less.

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October 11, 1949

### The Amino Acid Composition of Whole Wheat in Relation to its Protein Content

DEAR SIR:

During some recent studies in these laboratories the essential amino acid composition of 4 samples of different varieties of whole wheat has been determined by microbiological assay. The results suggest that the fraction of the total (Kjeldahl) nitrogen contributed by several of the amino acids may not be wholly independent of the nitrogen content of the sample, tending to be higher in samples of lower nitrogen content. This was most marked with arginine, lysine and valine, figures for which are given below. No such tendency was noted with phenylalanine, histidine, tryptophane or tyrosine. None of the amino acids so far studied (the ten "essential" amino acids and tyrosine) showed the reverse tendency.

|                       | Nitrogen | Arginine* | Lyane* | Valine* |
|-----------------------|----------|-----------|--------|---------|
|                       | %        | %         | %      | %       |
| White English Wheat   | 1.58     | 10.2      | 4.12   | 3.56    |
| N. S. W. Australian   | 1.81     | 9.08      | 3.81   | 3.44    |
| New Crop Plate        | 2.27     | 8.94      | 3.46   | 3.32    |
| Tough No. 2 Manitoban | 2.43     | 8.75      | 3.39   | 3.15    |

\* Amino acid nitrogen as per cent of total nitrogen.

We have not so far determined the non-protein nitrogen content of our samples; variations therein might, of course, explain the results observed. Alternatively, it may be that the samples contain different proportions of the individual wheat proteins.

<sup>5</sup> Personal communication, Meade Harris, Wallace and Tiernan Company, Inc., Chicago, Illinois.



Some data recently published by McElroy *et al.* (1), in which the essential amino acids in nine samples of one variety each of wheat, barley and oats were compared, show some interesting points of similarity. Their figures show that there was a within-species variation of amino acid content which, in the case of wheat, was most evident for lysine, arginine, valine, isoleucine and histidine. Furthermore, as they point out, it appears that the fraction of the total nitrogen contributed by the nitrogen of lysine may decrease as the total N increases—a tendency that, their figures suggest, may apply to certain other amino acids also.

Thus there appears to be accumulating evidence that the crude protein of wheat samples lowest in nitrogen may contain rather higher proportions of several of the amino acids, suggesting that there are qualitative, as well as quantitative, differences in protein content.

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October 28, 1949

#### Literature Cited

1. McELROY, L. W., CLANDININ, D. R., LOBAY, W., and PETHYBRIDGE, S. I. Nine essential amino acids in pure varieties of wheat. *J. Nutrition* **37**: 329–336 (1949).

## BOOK REVIEWS

**Outlines of Biochemistry.** By Ross Aiken Gortner. Third Edition edited by Ross Aiken Gortner, Jr. and Willis Alway Gortner. 1078 pp. John Wiley & Sons, Inc., New York and Chapman & Hall, Ltd. London. Price \$7.50.

*Outlines of Biochemistry* has long been regarded as a classic in its field. The appearance of this revised edition will be welcomed by biochemists everywhere. It was made possible through the efforts of a number of authorities on various phases of biochemistry, nearly all of whom were former students or colleagues of the late chief of the Division of Agricultural Biochemistry at the University of Minnesota.

While many advances have been made in the eleven years since the last revision, these have been incorporated without greatly altering the general form and size of the book. Subject matter is divided into six sections dealing with colloids, proteins, carbohydrates and related substances, lipids and essential oils, plant pigments and biochemical regulators. Most of the chapter headings are the same as formerly, but some have been combined, some completely rewritten, and three new chapters included.

The section on colloids has been greatly condensed and relatively little new material added. All the other sections have been expanded.

In the protein section a new chapter on protein denaturation has been added. The chapter on protein analysis now includes descriptions of ion exchange and chromatographic separation methods for separation of amino acid mixtures and microbiological techniques for amino acid analyses.

The carbohydrate section has been almost completely rewritten. The subject is developed in an excellent fashion and includes many citations of recent literature. Errors found in some structural formulas in the previous editions have been corrected. A new chapter on carbohydrate metabolism, a field in which remarkable progress has been made in the last few years, has been added.

Lipid metabolism is the subject of a new chapter in the lipid section. Recent findings on the mechanism and inhibition of fat oxidation are also reviewed.

Extensive revisions have been made in the last two sections to present up-to-date information on the chemistry of plant pigments, vitamins, hormones, and enzymes.

Contributors to the revision were Drs. S. I. Aronovsky, P. D. Boyer, D. R. Briggs, H. B. Bull, G. O. Burr, W. F. Geddes, R. A. Gortner, Jr., W. A. Gortner, H. O. Halvorson, W. M. Sandstrom, T. J. Schoch, and J. J. Willaman.

The third edition upholds the high standards set by the original author. It should be a valuable aid to every cereal chemist as well as to others working with biological materials.

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**Nutritional Data.** Published and distributed gratis by the H. J. Heinz Co., P. O. Box 57, Pittsburgh, Pa.

As a successor to "Nutritional Charts," "Nutritional Data" will be welcomed by the large number of people working in the medical and biological professions, and by nutritionists and dietitians who have valued the previous 12 editions of "Nutritional Charts" as a ready source of reference. "Nutritional Data" is a revision and expansion of its predecessor which contains much added material and retains many of the valuable features of the original publication. The subject matter, treated mostly in tabular form, is divided essentially into the following ten sections: Vitamins, The Essential Elements, Proteins and Amino Acids, the Availability of Nutrients, Signs and Symptoms of Malnutrition, the Metabolism and Action of Foods, Human Dietary Requirements, Planning Diets for Good Nutrition, Tables of Food Composition and Nutritive Value, Nutritional Activities of H. J. Heinz Co., Suggestions for Further Reading. The section devoted to tables of food composition and nutritive values forms the backbone of this publication. This section will be particularly

valuable to dietitians, and it constitutes a convenient source of reference for others. Compared to previously published tables of food composition in "Nutritional Charts," these new tables have been revised to conform with the latest analytical data available. That such a revision is desirable is indicated by the fact that the analyses of what might be considered well-known staple foods, such as whole-wheat flour, oatmeal and peanut butter, given in the present issue are somewhat different from those previously listed. The expression of the mineral content of foods in terms of grams or milligrams per 100 grams of edible portion rather than in terms of percent is a distinct improvement. The columns giving the alkaline or acid effect of foods which were previously incorporated in the tables of food composition have been deleted. The descriptive and factual material presented in "Nutritional Data" is in general quite accurate and reliable. Perhaps it would be desirable if in the section on vitamins the deficiency signs and symptoms which are listed were more clearly identified with the species involved, particularly where specific symptoms have not been identified in human beings. While an effort in this direction has been made, the differentiation between symptomology in different species is not as clear-cut as might be desired. A few inaccuracies have been found in the text, but in general, they are not of a serious nature. For example, the statement made on page 31 to the effect that copper is necessary for conversion of iron into hemoglobin is not as yet supported by experimental evidence, nor should pantothenyl alcohol which is listed on page 45 as a pantothenic acid antagonist be classified in this manner inasmuch as it is readily available to human beings and oxidized by them to pantothenic acid.

The reviewer feels certain that there will be a great demand for "Nutritional Data" and that it will be a valuable reference to all the people interested in the field of nutrition. The sponsors of this publication and the editorial staff are making a fine contribution to better nutrition through the compilation and distribution of "Nutritional Data."

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## Cereal Chemistry

### EDITORIAL POLICY

*Cereal Chemistry* publishes scientific papers dealing with raw materials, processes, or products of the cereal industries, or with analytical procedures, technological tests, or fundamental research, related thereto. Papers must be based on original investigations, not previously described elsewhere, which make a definite contribution to existing knowledge.

*Cereal Chemistry* gives preference to suitable papers presented at the Annual Meeting of the American Association of Cereal Chemists, or submitted directly by members of the Association. When space permits, papers are accepted from other scientists throughout the world.

The papers must be written in English and must be clear, concise, and styled for *Cereal Chemistry*.

Manuscripts for publication should be sent to the Editor in Chief. Advertising rates may be secured from and subscriptions placed with the Managing Editor, University Farm, St. Paul 1, Minnesota. Subscription rates, \$7.50 per year. Foreign postage, 50 cents extra. Single copies, \$1.50; foreign, \$1.60.

Entered as second-class matter March 3, 1932, at the post office at Lancaster, Pa., under the Act of August 24, 1912. Acceptance for mailing at special rate of postage provided for in Section 1193, Act of October 3, 1917, authorized February 16, 1924.

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### SUGGESTIONS TO AUTHORS

**General.** From January 1, 1948, an abstract will be printed at the beginning of each paper instead of a summary at the end, references will be numbered to provide the option of citing by number only, and date of receipt, author's connections, etc., will be shown in footnotes. Except on these points, authors will find the last volume of *Cereal Chemistry* a useful guide to acceptable arrangements and styling of papers. "On Writing Scientific Papers for Cereal Chemistry" (*Trans. Am. Assoc. Cereal Chem.* 6: 1-22, 1948) amplifies the following notes.

Authors should submit two copies of the manuscript, typed double spaced with wide margins on 8½ by 11 inch white paper, and all original drawings or photographs for figures. If possible, one set of photographs of figures should also be submitted. Originals can then be held to prevent damage, and the photographs can be sent to reviewers.

**Titles and Footnotes.** Titles should be specific, but should be kept short by deleting unnecessary words. The title footnote shows "Manuscript received . . ." and the name and address of the author's institution. Author footnotes, showing position and connections, are desirable although not obligatory.

**Abstract.** A concise abstract of about 200 words follows title and authors. It should state the principal results and conclusions, and should contain, largely by inference, adequate information on the scope and design of the investigation.

**Literature.** In general, only recent papers need be listed, and these can often be cited more advantageously throughout the text than in the introduction. Long introductory reviews should be avoided, especially when a recent review in another paper or in a monograph can be cited instead.

References are arranged and numbered in alphabetical order of authors' names and show author, title, journal, volume, first and last pages, and year. The list is given at the end of the paper. Reference numbers must invariably be cited in the text, but authors' names and year may be cited also. Abbreviations for the names of journals follow the list given in *Chemical Abstracts* 40: I-CCIX, 1946.

**Organization.** The standard organization involves main sections for abstract, introduction, materials, methods, results, discussion, acknowledgments, and literature cited. Alternately, a group of related studies, each made with different materials or methods, may require a separate section for each study, with subsections for materials and methods, and for results, under each section. Center headings are used for main sections and italicized run-in headings for subsections, and headings should be restricted to these two types only.

**Tables.** Data should be arranged to facilitate the comparisons readers must make. Tables should be kept small by breaking up large ones if this is feasible. Only about eight columns of tabular matter can be printed across the page. Authors should omit all unessential data such as laboratory numbers, columns of data that show no significant variation, and any data not discussed in the text. A text reference can frequently be substituted for columns containing only a few data. The number of significant figures should be minimized. Box and side headings should be kept short by abbreviating freely; unorthodox abbreviations may be explained in footnotes, but unnecessary footnotes should be avoided. Leader tables without a number, main heading, or ruled lines are often useful for small groups of data.

Tables should be typed on separate pages at the end of the manuscript, and their position should be indicated to the printer by typing "(TABLE I)" in the appropriate place between lines of the text. (Figures are treated in the same way.)

**Figures.** If possible, all line drawings should be made by a competent draftsman. Traditional layouts should be followed: the horizontal axis should be used for the independent variable; curves should be drawn heaviest, axes or frame intermediate, and the grid lines lightest; and experimental points should be shown. Labels are preferable to legends. Authors should avoid identification in cut-lines to be printed below the figure, especially if symbols are used that cannot readily be set in type.

All drawings should be made about two to three times eventual reduced size with India ink on white paper, tracing linen, or blue-lined graph paper; with any other color, the unsightly mass of small grid lines is reproduced in the cut. Lettering should be done with a guide using India ink; and letters should be  $\frac{1}{16}$  to  $\frac{1}{8}$  inch high after reduction.

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**Editorial Style** A.A.C.C. publications are edited in accordance with *A Manual of Style*, University of Chicago Press, and *Webster's Dictionary*. A few points which authors often treat wrongly are listed below:

Use names, not formulas, for text references to chemical compounds. Use plural verbs with quantities (6.9 g. were). Figures are used before unit abbreviations (3 ml.), and % rather than "per cent" is used following figures. All units are abbreviated and followed by periods, except units of time, which are spelled out. Repeat the degree sign ( $5^{\circ}$ - $10^{\circ}$  C.). Place 0 before the decimal point for correlation coefficients ( $r = 0.95$ ). Use \* to mark statistics that exceed the 5% level and \*\* for those that exceed the 1% level; footnotes explaining this convention are no longer required. Type fractions on one line if possible, e.g.,  $A/(B + C)$ . Use lower case for farinograph, mixogram, etc., unless used with a proper name, i.e., Brabender Farinograph. When in doubt about a point that occurs frequently, consult the Style Manual or the Dictionary.

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## THE ENVIRONMENTAL AND AGRONOMICAL FACTORS INFLUENCING THE THIAMINE, RIBOFLAVIN, NIACIN, AND PANTOTHENIC ACID CONTENT OF WHEAT, CORN, AND OATS<sup>1</sup>

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### ABSTRACT

Data compiled on the thiamine, riboflavin, niacin, and pantothenic acid contents of wheat, corn, and oats grown on three types of soil fertility experiments during several seasons were subjected to statistical analysis. Interpretation as to the effect of season (year in which crop was grown), lime, and various types of fertilizers were made on the basis of trends or statistically significant differences.

In the five-year rotation fertility experiment, season affected the thiamine and riboflavin content of wheat, corn, and oats, the niacin content of corn and oats, and the pantothenic acid content of corn.

Liming the soil in the five-year rotation experiment increased the thiamine content of wheat, corn, and oats, and the niacin and pantothenic acid content of wheat. Liming the soil also decreased the hull and niacin content of oats in 1944 (dry year). This decrease was less significant where a complete fertilizer (NPK) was used in comparison with single fertilizers (N or P or K).

The effect of fertilizers (other than lime) on the vitamin content of wheat was selective. Phosphorus alone appears to have increased the thiamine content, while nitrates increased the niacin and nitrates and potash in combination increased the pantothenic acid content of wheat. Potash alone appears to have decreased the thiamine content of wheat.

Nitrates alone significantly increased the thiamine content of oats. No other fertilizer effect, other than lime, was apparent.

The thiamine content of corn appears to have been decreased by potash as a single fertilizer. These results were apparent for two years. The thiamine content of corn grown during a dry season (1944) was less than the thiamine content of corn grown during a normal season.

Season affected the thiamine content of wheat and oats in the continuous culture experiment, but, unlike that found in the five-year rotation experiment, the thiamine content of corn was not affected. Wheat grown in a

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normal year (1948) had a higher riboflavin content than wheat grown in a wet year (1947). Reverse results were found in oats.

Liming the soil did not significantly increase the thiamine content of oats nor increase the thiamine content of corn. These results are the reverse of those found in the five-year rotation experiment.

The application of variable amounts of a 2-12-6 fertilizer produced no significant effect on the vitamin B complex content of wheat. Wheat grown on the "rate of fertilization experiment," of recent origin, had a higher average thiamine, riboflavin, niacin, and pantothenic acid content than wheat grown in the five-year rotation experiment and a greater niacin content than wheat grown in the continuous culture experiment.

Plants and their fruits supply almost the entire feed of animals and a large part of the food for man. Any information concerning factors which influence the value of plants and their fruits (grains) as a source of nutrients is of great nutritional and national importance.

It has been established that the application of certain fertilizers to the soil affects the composition of wheat, Ames (1, 2); Hunt (7). The question arises as to whether there is a correlation between the members of the vitamin B-complex and the changes in the other chemical components of the grain. Some investigators have observed that the vitamin B content is correlated with the phosphorus content of the grain, Voegtlin and Meyers (17), and that wheat grown on a soil fertilized with phosphate had a higher vitamin B content than wheat grown without phosphate fertilizer, Hunt (7). Rowlands and Wilkinson (12) have shown that soils treated with barnyard manure produced seeds of a higher vitamin B content than those produced with artificial commercial fertilizers. Since the above observations were made, vitamin B has been found to be a complex and nothing is known as to what factor or factors of the complex may be influenced by such soil treatment.

Schultz, Atkin, and Frey (14) have shown that there are significant differences in the thiamine content of different varieties of the same cereal and that there are indications that regional differences may affect the thiamine content of a single variety. Downs and Cathcart (5) found that hard wheats had a higher thiamine content ( $7.1 \mu\text{g./g.}$ ) than soft wheats ( $6.1 \mu\text{g./g.}$ ). Nordgren and Andrews (11) observed that location had a much greater effect than variety on the thiamine content of spring wheat, and that the thiamine content was correlated with the ash content. Johansson and Rich (9) found a large variability ( $2.9$  to  $8.0 \mu\text{g./g.}$ ) in the thiamine content of wheat and suggest that this variation may be due to soil composition, climatic conditions, and variety, while Whiteside and Jackson (18) reported a significant difference in the thiamine content of different varieties of spring wheat and stated that location and year also had a significant effect. Hoffer, Alcock, and Geddes (6) analyzed Canadian spring wheats from

three provinces and reported a range in thiamine from 2.9 to 6.3  $\mu\text{g./g.}$ , with an average of 4.56  $\mu\text{g./g.}$  They found a significant positive correlation between thiamine content and protein. Teply, Strong, and Elvehjem (16) have reported that the environmental differences under which wheat is grown influence the niacin, pantothenic acid, and pyridoxine content, but that the effect on each of the three vitamins was not in the same order. Knox, Heller, and Sieglinger (10) found that sorghum grains vary but little in riboflavin and pantothenic acid, while niacin varied as much as 100%. Hunt, Ditzler, and Bethke (8), and Ditzler, Hunt, and Bethke (4) have shown that both hereditary and environmental factors affected the niacin and pantothenic acid content of corn hybrids and some related inbred lines. Pantothenic acid was much more subject to environmental influences than was niacin.

Long range cultural experiments inaugurated at the Ohio Agricultural Experiment Station in 1893 and in 1937 offered an excellent opportunity to study the effect of soil treatment with fertilizers and lime on the vitamin B-complex content of wheat, corn, and oats. If soil treatment affects the vitamin B-complex content of grains the fact should become evident in these types of experiments.

### Materials and Methods

The types of experiments from which samples were gathered for assay were as follows:

(1) *Five-Year Rotation Fertility.* This experiment was inaugurated in 1893. The order of crops was corn, wheat, oats, and two years of forage crops. Samples were assayed from two crops of each of the cereal grains. The fertilizer treatments for each grain are shown in

TABLE I  
VARIANCE FOR THIAMINE, RIBOFLAVIN, NIACIN, AND PANTOTHENIC ACID  
CONTENTS OF WHEAT (FIVE-YEAR ROTATION EXPERIMENT)

| Source of variance       | Degrees of freedom | Thiamine | Riboflavin | Niacin  | Pantothenic acid |
|--------------------------|--------------------|----------|------------|---------|------------------|
| Year                     | 1                  | 31.38**  | 48.19**    | 3.38    | 0.08             |
| Lime                     | 1                  | 35.50**  | .13        | 22.04** | 18.85**          |
| Fertilizer               | 8                  | 3.75*    | .42        | 6.71*   | 4.56*            |
| Year $\times$ lime       | 1                  |          | 1.65       | 1.00    | 3.92             |
| Year $\times$ fertilizer | 8                  | 3.00     | 1.61       | 7.22**  | 2.68             |
| Lime $\times$ fertilizer | 8                  | 1.63     | .38        | .90     | 2.42             |
| Error                    | 8                  | .08      | .0069      | 2.16    | .75              |
| Total                    | 35                 |          |            |         |                  |

\* Significant.

\*\* Highly significant.



TABLE II  
EFFECT OF FERTILIZER TREATMENT ON THE THIAMINE, RIBOFLAVIN, NIACIN, AND PANTOTHENIC ACID CONTENT OF WHEAT  
FIVE YEAR ROTATION (MICROGRAMS PER GRAM)

| Plot No.                                  | Fertilizer treatment—pounds per acre                                                                                                        | Thiamine   |            | Riboflavin   |              | Niacin       |              | Pantothenic acid |              |
|-------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------|------------|------------|--------------|--------------|--------------|--------------|------------------|--------------|
|                                           |                                                                                                                                             | 1945       | 1946       | 1945         | 1946         | 1945         | 1946         | 1945             | 1946         |
| 2                                         | Superphosphate, 160<br>Superphosphate, 160+lime                                                                                             | 3.9<br>4.5 | 3.9<br>3.9 | 0.76<br>0.77 | 0.91<br>0.93 | 41.3<br>48.3 | 40.3<br>41.1 | 9.1<br>11.3      | 8.6<br>7.7   |
| 3                                         | Muriate of potash, 100<br>Muriate of potash, 100+lime                                                                                       | 3.1<br>3.4 | 2.6<br>3.7 | 0.74<br>0.81 | 0.96<br>0.87 | 44.0<br>45.3 | 39.5<br>42.7 | 9.2<br>11.8      | 10.3<br>9.5  |
| 4                                         | No treatment (check plot)<br>No treatment (check plot)+lime                                                                                 | 2.6<br>3.4 | 3.6<br>4.2 | 0.82<br>0.63 | 0.89<br>0.89 | 42.0<br>46.3 | 39.7<br>43.8 | 8.9<br>13.5      | 9.3<br>13.6  |
| 5                                         | Nitrate of soda, 160<br>Nitrate of soda, 160+lime                                                                                           | 2.8<br>3.7 | 3.5<br>4.7 | 0.71<br>0.84 | 0.97<br>0.78 | 46.3<br>48.0 | 42.8<br>47.4 | 8.5<br>10.6      | 10.6<br>11.4 |
| 6                                         | Superphosphate, 160; nitrate of soda, 160<br>Superphosphate, 160; nitrate of soda, 160+lime                                                 | 2.9<br>3.0 | 3.8<br>4.1 | 0.77<br>0.88 | 0.89<br>0.83 | 42.0<br>44.7 | 44.7<br>45.3 | 8.5<br>9.2       | 10.0<br>8.8  |
| 8                                         | Superphosphate, 160; muriate of potash, 100<br>Superphosphate, 160; muriate of potash, 100+lime                                             | 2.2<br>3.5 | 3.4<br>4.2 | 0.71<br>0.73 | 0.91<br>0.94 | 36.3<br>40.3 | 41.5<br>41.0 | 10.6<br>10.1     | 9.2<br>10.4  |
| 9                                         | Muriate of potash, 100; nitrate of soda, 160<br>Muriate of potash, 100; nitrate of soda, 160+lime                                           | 3.1<br>3.6 | 3.9<br>4.6 | 0.67<br>0.81 | 1.02<br>1.03 | 39.3<br>38.7 | 43.9<br>44.4 | 9.5<br>11.9      | 12.9<br>13.8 |
| 11                                        | Superphosphate, 160; muriate of potash, 100; nitrate of soda, 160<br>Superphosphate, 160; muriate of potash, 100; nitrate of soda, 160+lime | 3.4<br>3.8 | 3.7<br>4.3 | 0.67<br>0.83 | 1.03<br>0.99 | 38.3<br>41.7 | 43.7<br>46.2 | 10.6<br>11.5     | 8.7<br>10.8  |
| 17                                        | Superphosphate, 160; muriate of potash, 100; nitrate of soda, 80<br>Superphosphate, 160; muriate of potash, 100; nitrate of soda, 80+lime   | 3.3<br>3.7 | 3.9<br>3.4 | 0.70<br>0.66 | 1.02<br>1.11 | 37.0<br>38.3 | 42.9<br>43.4 | 9.8<br>11.2      | 10.9<br>10.7 |
|                                           | Average                                                                                                                                     | 3.3        | 3.9        | 0.75         | 0.94         | 42.1         | 43.0         | 10.3             | 10.4         |
| Minimum significant difference—Plot means |                                                                                                                                             | 0.41       | 0.12       | 0.12         | 0.18         | 2.13         | 1.26         | 0.99             | 0.58         |
| Yearly means                              |                                                                                                                                             | 0.19       |            |              |              |              |              |                  |              |

TABLE III  
EFFECT OF FERTILIZER TREATMENT ON THE THIAMINE, RIBOFLAVIN, NIACIN, AND PANTOTHENIC ACID CONTENT OF CORN  
FIVE-YEAR ROTATION EXPERIMENT (MICROGRAMS PER GRAM)

| Plot No.                                   | Fertilizer treatment—pounds per acre                                   | Thiamine |      | Riboflavin |      | Niacin |      | Pantothenic acid |      |
|--------------------------------------------|------------------------------------------------------------------------|----------|------|------------|------|--------|------|------------------|------|
|                                            |                                                                        | 1944     | 1948 | 1944       | 1948 | 1944   | 1948 | 1944             | 1948 |
| 2                                          | Superphosphate, 80                                                     | 3.2      | 5.3  | 0.97       | 1.14 | 20.7   | 24.8 | 5.7              | 8.6  |
|                                            | Superphosphate, 80+lime                                                | 4.2      | 6.6  | 0.83       | 1.05 | 20.7   | 24.8 | 5.3              | 7.8  |
| 3                                          | Muriate of potash, 80                                                  | 2.9      | 3.8  | 0.87       | 1.19 | 21.2   | 21.9 | 5.2              | 8.8  |
|                                            | Muriate of potash, 80+lime                                             | 3.1      | 5.2  | 0.77       | 1.22 | 20.7   | 23.3 | 4.7              | 6.0  |
| 4                                          | No treatment—check plot                                                | 3.0      | 5.9  | 1.15       | 1.37 | 21.5   | 23.7 | 7.0              | 8.9  |
|                                            | No treatment—check plot+lime                                           | 4.0      | 6.5  | 1.05       | 1.09 | 21.5   | 25.3 | 5.2              | 10.2 |
| 5                                          | Nitrate of soda, 160                                                   | 3.6      | 4.9  | 1.08       | 1.09 | 21.7   | 23.7 | 6.2              | 6.7  |
|                                            | Nitrate of soda, 160+lime                                              | 3.9      | 5.3  | 1.00       | 1.10 | 21.7   | 24.7 | 5.6              | 5.9  |
| 7                                          | No treatment—check plot                                                |          | 3.7  |            | 1.20 |        | 22.7 |                  | 6.6  |
|                                            | No treatment—check plot+lime                                           |          | 4.8  |            | 1.09 |        | 23.8 |                  | 8.4  |
| 8                                          | Superphosphate, 80; muriate of potash, 80                              |          | 5.5  |            | 0.98 |        | 24.9 |                  | 5.2  |
|                                            | Superphosphate, 80; muriate of potash, 80+lime                         |          | 6.9  |            | 1.05 |        | 25.0 |                  | 4.6  |
| 9                                          | Muriate of potash, 80; nitrate of soda, 160                            | 3.3      | 5.4  | 0.98       | 0.99 | 19.2   | 23.9 | 5.6              | 7.9  |
|                                            | Muriate of potash, 80; nitrate of soda, 160+lime                       | 4.3      | 6.4  | 0.93       | 1.03 | 20.7   | 24.5 | 5.2              | 4.1  |
| 10                                         | No treatment—check plot                                                |          | 4.3  |            | 1.41 |        | 24.6 |                  | 4.8  |
|                                            | No treatment—check plot+lime                                           |          | 5.2  |            | 1.06 |        | 25.5 |                  | 7.7  |
| 11                                         | Superphosphate, 160; muriate of potash, 100; nitrate of soda, 160      | 3.7      | 4.8  | 1.08       | 1.05 | 20.7   | 24.9 | 6.0              | 5.3  |
|                                            | Superphosphate, 160; muriate of potash, 100; nitrate of soda, 160+lime | 4.6      | 6.3  | 1.00       | 1.09 | 25.0   | 23.9 | 5.3              | 7.3  |
| 17                                         | Superphosphate, 80; muriate of potash, 80; nitrate of soda, 160        | 3.8      | 4.1  | 1.03       | 0.99 | 20.7   | 26.0 | 6.0              | 7.0  |
|                                            | Superphosphate, 80; muriate of potash, 80; nitrate of soda, 160+lime   | 4.7      | 4.9  | 1.08       | 1.04 | 21.7   | 25.7 | 4.9              | 8.6  |
|                                            | Average                                                                | 3.7      | 5.3  | 0.99       | 1.12 | 21.3   | 24.4 | 5.6              | 6.8  |
| Minimum significance difference—Plot means |                                                                        | 0.38     |      | 0.14       |      | 1.8    |      | 1.8              |      |
| Yearly means                               |                                                                        | 0.06     |      | 0.07       |      | 0.9    |      | 0.9              |      |

TABLE IV  
EFFECT OF FERTILIZER TREATMENT ON THE THIAMINE, RIBOFLAVIN, NIACIN, AND PANTOTHENIC ACID CONTENT OF OATS  
FIVE YEAR ROTATION (MICROGRAMS PER GRAM)

| Plot No.                                  | Fertilizer treatment—pounds per acre                                   | Thiamine |      | Riboflavin |      | Niacin |      | Pantothenic acid |      |
|-------------------------------------------|------------------------------------------------------------------------|----------|------|------------|------|--------|------|------------------|------|
|                                           |                                                                        | 1944     | 1945 | 1944       | 1945 | 1944   | 1945 | 1944             | 1945 |
| 2                                         | Superphosphate, 80                                                     | 4.0      | 4.0  | 1.37       | 1.18 | 14.3   | 7.3  | 7.8              | 7.0  |
|                                           | Superphosphate, 80 + lime                                              | 4.5      | 5.3  | 1.25       | 1.25 | 10.3   | 6.6  | 7.3              | 8.5  |
| 3                                         | Muriate of potash, 80                                                  | 3.6      | 4.4  | 1.33       | 1.10 | 13.2   | 6.4  | 7.2              | 5.8  |
|                                           | Muriate of potash, 80 + lime                                           | 3.8      | 4.7  | 1.30       | 1.28 | 10.2   | 6.6  | 6.6              | 8.2  |
| 4                                         | No treatment (check plot)                                              | 3.5      | 4.4  | 1.47       | 1.12 | 13.4   | 6.4  | 6.8              | 5.7  |
|                                           | No treatment (check plot) + lime                                       | 5.1      | 4.9  | 1.37       | 1.19 | 9.8    | 7.6  | 7.2              | 5.2  |
| 5                                         | Nitrate of soda, 160                                                   | 4.1      | 5.6  | 1.32       | 1.27 | 13.9   | 6.5  | 8.2              | 5.4  |
|                                           | Nitrate of soda, 160 + lime                                            | 5.3      | 6.2  | 1.25       | 1.37 | 10.4   | 6.9  | 6.9              | 4.8  |
| 6                                         | Superphosphate, 80; nitrate of soda, 160                               | —        | 5.6  | —          | 1.24 | —      | 6.3  | —                | 4.1  |
|                                           | Superphosphate, 80; nitrate of soda, 160 + lime                        | —        | 5.8  | —          | 1.20 | —      | 7.1  | —                | 7.6  |
| 8                                         | Superphosphate, 80; muriate of potash, 80                              | —        | 4.0  | —          | 1.13 | —      | 6.6  | —                | 5.8  |
|                                           | Superphosphate, 80; muriate of potash, 80 + lime                       | —        | 5.8  | —          | 1.32 | —      | 6.8  | —                | 5.2  |
| 9                                         | Muriate of potash, 80; nitrate of soda, 160                            | 4.0      | 5.0  | 1.45       | 1.38 | 12.0   | 6.7  | 7.5              | 7.0  |
|                                           | Muriate of potash, 80; nitrate of soda, 160 + lime                     | 4.6      | 5.6  | 1.25       | 1.44 | 10.0   | 6.9  | 7.3              | 7.4  |
| 11                                        | Superphosphate, 80; muriate of potash, 80; nitrate of soda, 160        | 4.0      | 4.2  | 1.28       | 1.30 | 13.2   | 6.6  | 7.7              | 5.8  |
|                                           | Superphosphate, 80; muriate of potash, 80; nitrate of soda, 160 + lime | 4.2      | 4.4  | 1.16       | 1.39 | 11.7   | 7.1  | 7.9              | 7.8  |
| 17                                        | Superphosphate, 160; muriate of potash, 80; nitrate of soda, 80        | 3.5      | 4.2  | 1.22       | 1.16 | 13.0   | 6.6  | 7.0              | 7.5  |
|                                           | Superphosphate, 160; muriate of potash, 80; nitrate of soda, 80 + lime | 4.5      | 5.6  | 1.05       | 1.09 | 11.2   | 6.4  | 6.4              | 8.2  |
|                                           | Average                                                                | 4.2      | 5.1  | 1.29       | 1.24 | 11.9   | 6.7  | 7.3              | 6.2  |
| Minimum significant difference—Plot means |                                                                        | 0.5      | 0.04 | 0.8        | 1.0  |        |      |                  |      |
| Yearly means                              |                                                                        | 0.24     | 0.04 | 0.4        | 0.5  |        |      |                  |      |

Tables II, III, and IV. Where lime is indicated, one-half of each plot was limed to pH 7.0.

(2) *Continuous Culture.* Corn, wheat, and oats were grown continuously on separate but adjacent plots since 1893. One-half of each plot was limed to pH 7.0 on all grains until 1934, since that time the entire plots on which wheat was grown have been limed. The fertilizer treatments for each grain are shown in Tables VI, VII, and VIII. Samples assayed included wheat and oats grown during two years, and a small number of samples of corn grown during three years.

The soil underlying the plots used in the above experiments is Wooster silt loam.

(3) *Rate of Fertilization.* This experiment was inaugurated in 1937. The rotation of crops grown was corn, wheat, and two years of alfalfa. Samples of wheat grown during four years were assayed. All plots received a basic treatment of lime to pH 7.0. The fertilizer treatment is shown in Table IX. The underlying soil is Canfield silt loam.

The same varieties of wheat and oats were grown throughout the experiments. The same corn hybrid was used throughout the experiments with the exception of one year of the five-year rotation fertility experiment; this fact will be treated more fully in the discussion of results.

All samples were finely ground in a Wiley mill and stored in tightly stoppered glass containers in a dark room until assayed. The assays for niacin and pantothenic acid were made as outlined in a previous publication, Hunt *et al.* (8), using *Lactobacillus arabinosus* 17-5 as the test organism.

The samples for riboflavin assay were prepared according to the procedure of Cooperman and Elvehjem (3) and determined according to the method of Snell and Strong (1939), using *Lactobacillus casei* E as the test organism.

The method used for thiamine followed closely that of Sarett and Cheldelin (13), using *Lactobacillus fermentum* 36 as the test organism. The samples were subjected to enzymatic hydrolysis. Bacterial growth at the end of 16 to 18 hours was measured as turbidity, which was read directly on the galvanometer scale of the Coleman spectrophotometer, at a wave length of 540 m $\mu$ . The scale was first standardized with distilled water to read 100. Readings on duplicates of three sample aliquots were made in matched tubes. The density  $L$  ( $L = 2 - \log G$ ) was calculated from the galvanometer reading  $G$ . To obtain a standard curve,  $L$  values were plotted against concentrations of thiamine in aliquots of a standard solution.

TABLE V  
THE RELATIONSHIP BETWEEN WEIGHT OF OATS AND PERCENT HULLS, AND NIAICIN CONTENT OF OATS, GROATS, AND HULLS  
Oats 1944—Dry weather

| Plot No. | Fertilizer treatment—pounds per acre                                                                                                    | Weight<br>per bu. | Groats | Hulls | Niacin— $\mu\text{g./g.}$ |        |       |
|----------|-----------------------------------------------------------------------------------------------------------------------------------------|-------------------|--------|-------|---------------------------|--------|-------|
|          |                                                                                                                                         |                   |        |       | Oat grains                | Groats | Hulls |
|          |                                                                                                                                         | lb.               | pct.   | pct.  |                           |        |       |
| 2        | Superphosphate, 80<br>Superphosphate, 80+lime                                                                                           | 23                | 60.2   | 39.8  | 14.3                      | 12.0   | 18.2  |
|          |                                                                                                                                         | 30                | 66.6   | 33.4  | 10.3                      | 10.6   | 11.4  |
| 3        | Muriate of potash, 80<br>Muriate of potash, 80+lime                                                                                     | 25                | 63.4   | 36.6  | 13.2                      | 11.7   | 16.3  |
|          |                                                                                                                                         | 30                | 66.5   | 33.5  | 10.2                      | 9.7    | 11.2  |
| 4        | No treatment (check plot)<br>No treatment (check plot)+lime                                                                             | 23                | 62.8   | 37.2  | 13.4                      | 12.0   | 17.2  |
|          |                                                                                                                                         | 28                | 67.4   | 32.6  | 9.8                       | 9.7    | 10.5  |
| 5        | Nitrate of soda, 160<br>Nitrate of soda, 160+lime                                                                                       | 25                | 62.3   | 37.7  | 13.9                      | 10.5   | 17.5  |
|          |                                                                                                                                         | 28                | 66.6   | 33.4  | 10.4                      | 8.0    | 11.2  |
| 9        | Muriate of potash, 80; nitrate of soda, 160<br>Muriate of potash, 80; nitrate of soda, 160+lime                                         | 26                | 61.9   | 38.1  | 12.0                      | 10.2   | 13.7  |
|          |                                                                                                                                         | 29                | 69.0   | 31.0  | 10.0                      | 8.8    | 9.9   |
| 11       | Superphosphate, 80; muriate of potash, 80; nitrate of soda, 160<br>Superphosphate, 80; muriate of potash, 80; nitrate of soda, 160+lime | 27                | 66.6   | 33.4  | 13.2                      | 10.5   | 20.5  |
|          |                                                                                                                                         | 30                | 68.7   | 31.3  | 11.7                      | 9.5    | 16.2  |
| 17       | Superphosphate, 160; muriate of potash, 80; nitrate of soda, 80<br>Superphosphate, 160; muriate of potash, 80; nitrate of soda, 80+lime | 26                | 64.7   | 35.3  | 13.0                      | 10.7   | 19.0  |
|          |                                                                                                                                         | 28                | 66.5   | 33.5  | 11.2                      | 9.5    | 15.3  |
|          | Average—unlimed<br>Average—limed                                                                                                        | 25                | 63.1   | 36.9  | 13.0                      | 11.2   | 17.6  |
|          |                                                                                                                                         | 29                | 67.3   | 32.7  | 10.4                      | 9.4    | 12.4  |

TABLE V—Cont.

Oats 1945—Normal weather

| Plot No. | Fertilizer treatment—pounds per acre | Weight<br>per bu. | Groats       | Hulls        | Niacin— $\mu$ g./g. |            |            |
|----------|--------------------------------------|-------------------|--------------|--------------|---------------------|------------|------------|
|          |                                      |                   |              |              | Oat Grains          | Groats     | Hulls      |
|          |                                      | lb.               | <i>pcf.</i>  | <i>pcf.</i>  |                     |            |            |
| 2        | Fertilizer treatment, same as above  | 31.0<br>31.0      | 65.9<br>65.9 | 34.1<br>34.1 | 7.3<br>6.6          | 6.4<br>6.9 | 6.4<br>7.4 |
| 3        | Fertilizer treatment, same as above  | 31.5<br>28.5      | 67.4<br>63.2 | 32.6<br>36.8 | 6.4<br>6.6          | 7.1<br>6.4 | 7.2<br>8.0 |
| 4        | Fertilizer treatment, same as above  | 30.0<br>30.0      | 67.6<br>66.7 | 32.4<br>33.3 | 6.4<br>7.6          | 6.4<br>7.1 | 6.1<br>7.3 |
| 5        | Fertilizer treatment, same as above  | 31.5<br>30.0      | 67.9<br>62.7 | 32.1<br>37.3 | 6.5<br>6.9          | 6.6<br>7.3 | 6.5<br>8.1 |
| 9        | Fertilizer treatment, same as above  | 29.5<br>29.0      | 66.4<br>63.4 | 33.6<br>36.6 | 6.7<br>6.9          | 6.7<br>7.5 | 7.8<br>8.6 |
| 11       | Fertilizer treatment, same as above  | 30.0<br>29.5      | 68.8<br>67.8 | 31.2<br>32.2 | 6.6<br>7.1          | 5.8<br>7.8 | 5.8<br>8.7 |
| 17       | Fertilizer treatment, same as above  | 31.0<br>30.5      | 67.8<br>68.4 | 32.2<br>31.6 | 6.6<br>6.4          | 7.4<br>7.2 | 6.9<br>5.8 |
|          | Average—unlimed<br>Average—limed     | 30.6<br>29.8      | 67.4<br>65.4 | 32.6<br>34.6 | 6.7<br>6.8          | 6.7<br>7.1 | 6.6<br>7.7 |

TABLE VI  
EFFECT OF FERTILIZER TREATMENTS ON THE THIAMINE, RIBOFLAVIN, NIACIN, AND PANTOTHENIC ACID CONTENT OF WHEAT  
CONTINUOUS CULTURE (MICROGRAMS PER GRAM)

| Plot No.                                  | Fertilizer treatment—pounds per acre                                                                                                                        | Thiamine   |            | Riboflavin   |              | Niacin       |              | Pantothenic acid |              |
|-------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|------------|--------------|--------------|--------------|--------------|------------------|--------------|
|                                           |                                                                                                                                                             | 1947       | 1948       | 1947         | 1948         | 1947         | 1948         | 1947             | 1948         |
| 1                                         | No fertilizer (check plot)<br>No fertilizer (check plot) + lime (before 1934)<br>Superphosphate, 128; muriate of potash, 100; nitrate of soda, 160          | 3.0<br>3.5 | 4.4<br>4.8 | 1.13<br>1.15 | 1.23<br>1.32 | 46.3<br>42.5 | 48.7<br>50.6 | 13.1<br>13.4     | 13.4<br>13.4 |
| 2                                         | Superphosphate, 128; muriate of potash, 100; nitrate of soda, 160<br>Superphosphate, 128; muriate of potash, 100; nitrate of soda, 160 + lime (before 1934) | 3.3<br>3.6 | 4.7<br>4.5 | 1.25<br>1.15 | 1.16<br>1.22 | 42.7<br>43.3 | 45.1<br>45.7 | 11.8<br>10.5     | 11.7<br>11.2 |
| 3                                         | Superphosphate, 128; muriate of potash, 100<br>Superphosphate, 128; muriate of potash, 100 + lime (before 1934)                                             | 3.5<br>3.7 | 4.8<br>5.1 | 0.95<br>1.08 | 1.26<br>1.31 | 41.8<br>38.8 | 45.7<br>45.0 | 10.2<br>10.2     | 12.2<br>13.4 |
| 4                                         | No fertilizer (check plot)<br>No fertilizer (check plot) + lime (before 1934)                                                                               | 3.5<br>3.0 | 4.6<br>4.7 | 0.93<br>1.21 | 1.04<br>1.19 | 44.3<br>40.5 | 45.3<br>48.7 | 13.2<br>11.4     | 13.9<br>13.4 |
| 5                                         | Barnyard manure—2 1/2 tons<br>Barnyard manure—2 1/2 tons + lime (before 1934)                                                                               |            | 4.5<br>5.1 |              | 1.21<br>1.17 |              | 47.7<br>49.6 |                  | 13.2<br>13.8 |
| 6                                         | Barnyard manure—5 tons<br>Barnyard manure—5 tons + lime (before 1934)                                                                                       | 3.8<br>3.7 | 4.9<br>4.7 | 1.08<br>1.20 | 1.23<br>1.10 | 54.3<br>50.7 | 47.6<br>45.3 | 12.8<br>12.1     | 13.9<br>13.0 |
| 7                                         | No fertilizer (check plot)<br>No fertilizer (check plot) + lime (before 1934)                                                                               | 3.2<br>3.8 | 4.6<br>4.8 | 1.01<br>1.00 | 1.32<br>1.26 | 48.2<br>46.1 | 45.3<br>48.1 | 12.1<br>12.5     | 13.4<br>13.9 |
| 8                                         | Superphosphate, 256; muriate of potash, 200; nitrate of soda, 320<br>Superphosphate, 256; muriate of potash, 200; nitrate of soda, 320 + lime (before 1934) | 3.6<br>3.4 | 4.8<br>4.9 | 1.06<br>1.04 | 1.22<br>1.11 | 55.1<br>44.1 | 47.3<br>47.6 | 12.9<br>12.5     | 13.1<br>12.5 |
| 9                                         | Superphosphate, 256; muriate of potash, 200<br>Superphosphate, 256; muriate of potash, 200 + lime (before 1934)                                             | 3.7<br>3.3 | 4.9<br>4.6 | 1.15<br>1.03 | 1.18<br>1.29 | 50.2<br>45.8 | 43.7<br>43.4 | 12.3<br>11.6     | 12.5<br>12.7 |
| 10                                        | No fertilizer (check plot)<br>No fertilizer (check plot) + lime (before 1934)                                                                               | 3.3<br>3.3 | 4.4<br>3.9 | 1.00<br>1.09 | 1.14<br>1.15 | 53.6<br>46.4 | 48.4<br>47.3 | 13.5<br>13.1     | 14.4<br>13.1 |
|                                           | Average                                                                                                                                                     | 3.5        | 4.7        | 1.08         | 1.21         | 46.4         | 46.8         | 12.1             | 13.1         |
| Minimum significant difference—Plot means |                                                                                                                                                             | 0.35       |            | 0.14         |              | 4.00         |              | 0.80             |              |
| Yearly means                              |                                                                                                                                                             | 0.14       |            | 0.06         |              | 1.86         |              | 0.40             |              |

TABLE VII  
THE EFFECT OF FERTILIZERS ON THE THIAMINE, RIBOFLAVIN, NIACIN, AND PANTOTHENIC ACID CONTENT OF CORN  
CONTINUOUS CULTURE (MICROGRAMS PER GRAM)

| Plot No.                                  | Fertilizer treatment (lbs./acre)                                                                                                                    | Thiamine   |            |            | Riboflavin   |              |              | Niacin       |              |              | Pantothenic acid |            |            |
|-------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------|------------|------------|------------|--------------|--------------|--------------|--------------|--------------|--------------|------------------|------------|------------|
|                                           |                                                                                                                                                     | 1945       | 1946       | 1947       | 1945         | 1946         | 1947         | 1945         | 1946         | 1947         | 1945             | 1946       | 1947       |
| 1                                         | No fertilizer (check plot)<br>No fertilizer (check plot) + lime                                                                                     | 3.4<br>3.4 | 3.7<br>3.4 | 3.8<br>3.5 | 0.88<br>0.88 | 0.97<br>0.95 | 1.13<br>0.87 | 23.8<br>21.0 | 27.1<br>25.6 | 28.8<br>26.0 | 6.8<br>5.5       | 5.7<br>5.1 | 5.8<br>6.1 |
| 8                                         | Superphosphate, 256; muriate of potash, 200;<br>nitrate of soda, 320<br>Superphosphate, 256; muriate of potash, 200;<br>nitrate of soda, 320 + lime | 3.6<br>3.8 | 4.5<br>4.2 | 3.7<br>3.5 | 0.76<br>0.82 | 0.88<br>0.88 | 0.94<br>0.92 | 21.7<br>21.0 | 25.4<br>25.5 | 25.5<br>28.0 | 5.5<br>6.8       | 4.5<br>5.3 | 6.2<br>5.9 |
|                                           | Average                                                                                                                                             | 3.6        | 4.0        | 3.6        | 0.84         | 0.92         | 0.97         | 21.9         | 25.9         | 27.1         | 6.2              | 5.2        | 6.0        |
| Minimum significant difference—Plot means |                                                                                                                                                     | 0.06       |            |            |              | 0.07         |              |              | 1.30         |              |                  | 1.04       |            |
| Yearly means                              |                                                                                                                                                     | 0.07       |            |            |              | 0.09         |              |              | 1.60         |              |                  | 1.29       |            |



TABLE VIII  
EFFECT OF FERTILIZER TREATMENT ON THE THIAMINE, RIBOFLAVIN, NIACIN, AND PANTOTHENIC ACID CONTENT OF OATS  
CONTINUOUS CULTURE (MICROGRAMS PER GRAM)

| Plot No.                                  | Fertilizer treatment—pounds per acre                                                                                                        | Thiamine   |            | Riboflavin   |              | Niacin      |            | Pantothenic acid |              |
|-------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------|------------|------------|--------------|--------------|-------------|------------|------------------|--------------|
|                                           |                                                                                                                                             | 1947       | 1948       | 1947         | 1948         | 1947        | 1948       | 1947             | 1948         |
| 1                                         | No fertilizer—check plot<br>No fertilizer—check plot+lime                                                                                   | 5.0<br>5.9 | 5.8<br>6.3 | 3.73<br>2.98 | 0.97<br>1.02 | 10.5<br>9.4 | 8.5<br>9.0 | 13.7<br>15.7     | 10.1<br>11.1 |
| 2                                         | Superphosphate, 128; muriate of potash, 100; nitrate of soda, 160<br>Superphosphate, 128; muriate of potash, 100; nitrate of soda, 160+lime | 5.6<br>5.5 | 6.0<br>6.1 | 2.49<br>2.92 | 0.85<br>0.88 | 8.1<br>9.5  | 8.9<br>8.9 | 12.5<br>13.1     | 10.9<br>9.2  |
| 3                                         | Superphosphate, 128; muriate of potash, 100<br>Superphosphate, 128; muriate of potash, 100+lime                                             | 4.0<br>4.7 | 5.4<br>5.7 | 2.40<br>2.71 | 0.88<br>0.90 | 8.1<br>8.9  | 8.6<br>8.2 | 10.1<br>12.6     | 10.9<br>10.2 |
| 4                                         | No fertilizer—check plot<br>No fertilizer—check plot+lime                                                                                   | 5.4<br>5.1 | 6.1<br>6.2 | 3.72<br>3.07 | 0.99<br>0.91 | 10.1<br>9.9 | 8.2<br>8.9 | 14.1<br>11.1     | 9.4<br>10.0  |
| 5                                         | Barnyard manure—2.5 tons<br>Barnyard manure—2.5 tons+lime                                                                                   | 5.5<br>6.1 | 5.4<br>5.7 | 2.95<br>2.62 | 0.84<br>0.84 | 8.1<br>9.2  | 8.3<br>8.6 | 9.8<br>10.3      | 9.6<br>10.3  |
| 6                                         | Barnyard manure—5 tons<br>Barnyard manure—5 tons+lime                                                                                       | 4.0<br>5.5 | 5.7<br>5.5 | 2.24<br>2.62 | 0.92<br>0.85 | 9.1<br>9.1  | 8.5<br>8.9 | 10.0<br>9.9      | 10.5<br>8.8  |
| 7                                         | No fertilizer—check plot<br>No fertilizer—check plot+lime                                                                                   | 5.2<br>4.7 | 5.8<br>6.0 | 2.72<br>2.62 | 0.93<br>0.97 | 9.0<br>9.2  | 8.6<br>8.9 | 9.5<br>10.0      | 9.9<br>9.9   |
| 8                                         | Superphosphate, 256; muriate of potash, 200; nitrate of soda, 320<br>Superphosphate, 256; muriate of potash, 200; nitrate of soda, 320+lime | 5.6<br>6.1 | 6.5<br>6.0 | 2.16<br>2.13 | 0.95<br>1.05 | 8.1<br>9.8  | 8.5<br>7.5 | 9.1<br>9.7       | 9.2<br>11.8  |
| 9                                         | Superphosphate, 256; muriate of potash, 200<br>Superphosphate, 256; muriate of potash, 200+lime                                             | 4.1<br>4.2 | 4.1<br>4.5 | 2.42<br>2.62 | 0.91<br>0.95 | 7.7<br>8.8  | 8.2<br>8.7 | 7.2<br>8.4       | 6.6<br>12.6  |
| 10                                        | No fertilizer—check plot<br>No fertilizer—check plot+lime                                                                                   | 4.5<br>4.3 | 6.1<br>6.1 | 3.09<br>2.77 | 1.03<br>1.14 | 9.5<br>9.3  | 8.4<br>8.8 | 10.4<br>10.2     | 12.0<br>10.7 |
|                                           | Average                                                                                                                                     | 5.1        | 5.8        | 2.75         | 0.94         | 9.1         | 8.6        | 10.9             | 10.2         |
| Minimum significant difference—Plot means |                                                                                                                                             | 0.30       | 0.30       | 0.30         | 0.14         | 0.89        | 1.87       | 0.85             |              |
| Yearly means                              |                                                                                                                                             | 0.14       | 0.14       | 0.14         |              | 0.40        |            |                  |              |

TABLE IX  
EFFECT OF RATE OF FERTILIZATION ON THE VITAMIN B-COMPLEX OF WHEAT  
(MICROGRAMS PER GRAM)

| Plot No.             | 1          | 3                   | 6                   | 9                    |      |
|----------------------|------------|---------------------|---------------------|----------------------|------|
| Fertilizer treatment | None +lime | 200#2-12-6<br>+lime | 500#2-12-6<br>+lime | 1000#2-12-6<br>+lime | Av   |
| <i>lbs /acre</i>     |            |                     |                     |                      |      |
| Thiamine             |            |                     |                     |                      |      |
| 1944                 | 3.2        | 3.4                 | 3.8                 | 3.4                  | 3.5  |
| 1945                 | 5.4        | 5.1                 | 5.3                 | 5.3                  | 5.3  |
| 1946                 | 4.7        | 4.9                 | 4.8                 | 5.0                  | 4.9  |
| 1947                 | 4.2        | 3.8                 | 3.6                 | 3.2                  | 3.7  |
| Av.                  | 4.4        | 4.3                 | 4.4                 | 4.2                  | 4.3  |
| Riboflavin           |            |                     |                     |                      |      |
| 1944                 | 1.04       | 0.94                | 1.13                | 0.82                 | 0.98 |
| 1945                 | 1.16       | 1.28                | 1.18                | 1.21                 | 1.21 |
| 1946                 | .89        | .96                 | 1.15                | .91                  | .98  |
| 1947                 | 1.09       | 1.08                | 1.10                | 1.15                 | 1.10 |
| Av.                  | 1.05       | 1.07                | 1.23                | 1.02                 | 1.08 |
| Niacin               |            |                     |                     |                      |      |
| 1944                 | 60.2       | 57.0                | 52.0                | 51.2                 | 55.1 |
| 1945                 | 58.7       | 60.3                | 59.7                | 56.3                 | 58.8 |
| 1946                 | 43.8       | 48.5                | 49.8                | 51.8                 | 48.5 |
| 1947                 | 51.7       | 46.3                | 44.3                | 44.0                 | 46.6 |
| Av.                  | 53.6       | 53.0                | 51.5                | 50.8                 | 52.2 |
| Pantothenic acid     |            |                     |                     |                      |      |
| 1944                 | 12.0       | 13.2                | 12.8                | 11.8                 | 12.8 |
| 1945                 | 12.3       | 12.3                | 10.9                | 10.5                 | 11.5 |
| 1946                 | 10.5       | 10.3                | 11.7                | 10.4                 | 10.7 |
| 1947                 | 14.4       | 12.9                | 11.5                | 12.0                 | 12.7 |
| Av.                  | 12.3       | 12.2                | 11.7                | 11.2                 | 11.8 |

Minimum significant difference—yearly means: Thiamine, 0.43, Riboflavin, 0.14, Niacin, 5.53, Pantothenic Acid, 1.35.

A reference sample was used in each set of assays and for each vitamin. If the assay of the reference sample was within 10% of its known vitamin content, the assay values of the unknown samples were accepted.

All of the data were subjected to statistical analysis. Interpretations of results are based on statistically significant differences in vitamin content.

### Results and Discussion

*Five-Year Rotation Fertility Experiment.* The fertilizer treatments consisted of nitrogen, phosphorus, and potash additions and various combinations of these elements, all with and without lime and no treatment. Each plot received a different fertilizer treatment. Analysis of variance was made on the data. As an illustration, Table I shows the variance for the vitamins of wheat. Each vitamin was tested for variance due to year, lime, fertilizer, and interactions of each

with the other. In the cases where the data of only one year were available from a given plot, that plot was omitted from the analysis of variance test.

*Wheat.* The data for wheat are presented in Table II. The results show that season (i.e., the year the samples were grown) influenced the thiamine and riboflavin contents. The wheat grown in 1946 had significantly higher thiamine and riboflavin contents than that grown in 1945. This was true irrespective of the fertilizer treatment of the soil. Season had no effect on the niacin and pantothenic acid contents of wheat. The most outstanding and at the same time constant result was a higher thiamine, niacin, and pantothenic acid content produced by the application of lime. This effect (Table II) was highly significant for both years. The effects of fertilizers on thiamine were variable. The data indicate that the applications of superphosphate and potash as single treatments resulted in a slight increase and decrease, respectively, in thiamine content. This was true for both years of the study. Nitrate fertilizer alone significantly increased the niacin content of wheat, and no fertilizer treatment (check plot), as well as nitrate and potash in combination, produced wheat of the highest pantothenic acid content.

Hunt (7) reported that liming of the soil increased the phosphorus and magnesium contents of wheat. The data in this study show that liming of the soil increased the thiamine content, suggesting that there may be a close association between phosphorus and magnesium and the thiamine content of wheat. Since no mineral analyses were made on corn or oats in this or other studies, it is not known whether or not this association might occur in these cereals.

*Corn.* Season had a significant effect on the thiamine, riboflavin, niacin, and pantothenic acid contents of corn (Table III). The 1948 crop was higher in all four vitamins than the 1944 crop. The hybrid Ohio W17 was grown in 1944 and Ohio W36 was grown in 1948. In view of this fact, differences due to season cannot be clearly determined since the two hybrids may have responded to the seasonal variations in a different manner; however, the inference is that season had a significant effect.

Weather records show that 1944 was very dry during the growing season, while 1948 was termed a normal growing period with a normal rainfall. As with wheat, lime increased the thiamine content significantly while potash decreased the thiamine content significantly. Lime decreased riboflavin and had no effect on niacin and pantothenic acid.

*Oats.* The data on oats show further evidence that season affected the thiamine content of the cereals studied (Table IV). The 1945

crop was significantly higher in thiamine than the 1944 crop. Liming of the soil increased the thiamine content. Nitrate fertilizer alone increased the thiamine content of oats. Lime decreased riboflavin in 1944 and increased it in 1945; these effects were slight, quantitatively, but statistically significant because of consistency throughout the plots.

The 1944 crop of oats was higher in niacin than the 1945 crop by approximately 75%. This difference was highly significant. In 1944, the limed plots yielded oats consistently lower in niacin than that from the unlimed plots; in 1945, the effect of lime was not consistent. The fertilizer treatments were the same for both years, so that the differences in the niacin content and in the effect of lime could not be accounted for on the basis of fertilizer. In search of a possible explanation, other factors were investigated. Weather records showed that 1944 was a dry year and 1945 had normal rainfall. Weight per bushel and hull content of oats were determined (Table V). In 1944, liming the soil was shown to be associated not only with lower niacin but also with higher weight per bushel and obviously lower hull content. The niacin content of hulls was higher than that of groats. In 1945, the effect of lime was not consistent for niacin, weight per bushel, or hull content. From the foregoing facts, it was concluded that factors which increase hull content of oats also increase the niacin content. The action of lime during a dry year had a decreasing effect on niacin with related effects on weight per bushel and hull content. It is interesting to note that differences between the hull and niacin content of oats when grown on the limed and unlimed plots were less where a complete fertilizer (NPK) was used than where a single fertilizer (N or P or K) was applied to the soil (see Table V). This appears to apply only under extreme weather (dry) conditions.

The fact that liming the soil increased the thiamine content of all three cereals studied would lead to a general statement that liming the type of soil on which the crops were grown for this study would increase the thiamine content of wheat, corn, and oats, irrespective of season and the fertilizer applied to the soil.

*Continuous Culture Experiment.* The fertilizer treatments consisted of phosphate and potash in combination, complete, barnyard manure, and no treatment. In wheat and oats, samples from two or more replications of each fertilizer treatment were assayed, with the exception of barnyard manure on wheat in 1947, of which one sample only was assayed. Analysis of variance was performed to test variance due to year, lime, and plot, and interactions of each with the other. Variance due to plot may have been the result of fertilizer treatment as well as the variance between replications in the same field. In order to determine the significance of differences due to fertilizer, group

comparisons (t values) were made on the grouped samples receiving a given fertilizer with other groups on different fertilizer treatments.

*Wheat.* As in the five-year rotation fertility experiment, season affected the thiamine and riboflavin, and, in addition, the pantothenic acid content (Table VI). The wheat grown in 1948 (normal weather) had higher thiamine, riboflavin, and pantothenic acid contents than wheat grown in 1947 (high rainfall in April, May, and June). Comparison of fertilizer treatments for both years shows that the check plot (no fertilizer) produced wheat of the highest pantothenic acid content. As previously noted, one-half of each plot was limed until 1934, after which the entire plot was limed. At the time the samples were collected, therefore, both ends of the plots were receiving the same lime treatment. Any variations in the vitamin content of wheat grown on the two ends of the plots, as noted, may be due to a residual effect of liming previous to 1934 (Table VI).

*Corn.* The data in Table VII show that the niacin content of corn was influenced by season. No other effects of fertilizers, including lime, were observed. This does not agree with the results obtained in the five-year rotation experiment.

*Oats.* Assay results (Table VIII) show that the thiamine content of oats is affected by the season and that complete fertilizer (NPK) barnyard manure, and no fertilizer (check plot) produced oats of higher thiamine content than phosphorus and potash in combination. Liming the soil increased the thiamine content of oats but the effect was not as significant as that found in the five-year rotation experiment. The effect of liming on the niacin content of oats was not so discernible as in the five-year rotation experiment. A difference between a dry and wet and normal season may be the cause of this difference.

The riboflavin and niacin contents of this cereal were influenced by year (Table VIII). The oats grown in 1947 were much higher in riboflavin and slightly higher in niacin than that grown in 1948. This difference in effect of season appears to be due to the seasonal (weather) effect on that part of the grains in which the greater portion of the vitamin was or is being stored. Due to excess rainfall, the 1947 crop was very light in weight (about 20 pounds per bushel), which would indicate a high hull content. As was stated previously, light weight, high hull oats grown in the dry year, 1944, on the five-year rotation fertility experiment, were found to be correlated with a considerable increase in niacin. In the continuous culture experiment, the variation due to season was not nearly so marked.

These differences in magnitude of the effects of lime and season may have been due to type of experiment.

*Rate of Fertilization Experiment. Wheat.* The results are shown in Table IX. As in the other two experiments, the results show that season had a significant effect on the thiamine, riboflavin, niacin, and pantothenic acid contents of wheat. There was no consistent effect of extreme seasons (excessive rainfall or dryness) on the vitamins.

The rate of fertilizer application to the soil in this experiment had no significant effect on the vitamin B-complex content. It is also noted that there was no difference between the vitamin content of wheat from the check and fertilized plots.

The wheat grown on the plots in this experiment (of a more recent origin) had higher average thiamine, riboflavin, niacin, and pantothenic acid contents than the wheat grown on the five-year rotation fertility experiment, and the niacin content of the continuous culture experiment. This difference may be accounted for by the type of experiment and the length of time the experiment has been in operation.

The effect of single fertilizer ingredients and combinations of single fertilizer ingredients, as reported in this study, have been stated with caution due to lack of replication of plots in the five-year rotation experiment. However, it was hoped that year duplication would suffice in a manner to make the data valuable. The statistical analyses have indicated the results. Replicate plots in the continuous culture experiment showed some variation. The fact that all cereals from all experiments were not available the same season (year) no doubt caused variations in vitamin content which cannot be properly evaluated statistically. It should be noted, however, that the results, while not always highly significant, are good indications of the effect of fertilizers on the vitamin B-complex of cereals as stated.

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## AMINO ACID CONTENT OF VARIOUS WHEAT VARIETIES

### I. CYSTINE, LYSINE, METHIONINE, AND GLUTAMIC ACID<sup>1</sup>

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and G. D. MILLER

#### ABSTRACT

Differences in "microbiologically apparent" cystine and methionine with respect to environment were observed for several varieties of hard red winter wheat grown during one crop year. No differences in lysine or glutamic acid with respect to environment were observed. Likewise, no differences among wheat varieties were found for either cystine, lysine, methionine, or glutamic acid. All values are based on total wheat protein. There was a significant difference in per cent cystine for samples grown in 1946 and 1947. The wheat grown in 1947 contained the most cystine and also required longer mixing for optimum dough development. Thus there may be a relationship between per cent cystine and dough mixing time as influenced by environment. The longer mixing varieties tended to reflect a greater change in mixing requirement for a small change in cystine content. The nutritional value of wheat protein may be affected by environment due to variation in cystine and methionine content in the protein.

The protein content of wheat is variable and may be affected by various environmental factors including soil type, fertilizer treatment, and weather. Little is known, however, concerning the relationship of these factors to the relative amino acid composition of the protein.

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The development of microbiological as well as highly specific colorimetric methods for amino acid assay has made practicable the estimation of the relative proportions of specific amino acids in natural products. Although the assessment of validity of the assays necessitates a considerable number of tests, the results lend themselves to statistical treatment.

Certain amino acids have received more attention than others. Greaves and Bracken (9) have shown that different varieties of wheat and wheat receiving various cultural treatments differ in both total sulfur and total nitrogen. These workers found a highly significant correlation between total sulfur and total nitrogen and obtained evidence that all of the sulfur in wheat is in the organic form. Gubler and Greaves (10) found a variation in cystine content between varieties as well as between wheats grown under varying cultural conditions. A positive correlation was observed between cystine and sulfur as well as between cystine and nitrogen content. Approximately 50% of the total sulfur of wheat was found to be in the form of cystine. Csonka (5) found that the protein of Marquis wheat contained 1.43% cystine while Tenmarq and Fulhio contained 1.16 and 1.18% respectively. Little work has been reported on the influence of variety and environment on the relative amino acid content of wheat proteins.

Osborne and Mendel (16) concluded that "Wheat proteins considered in their entirety are adequate for promoting normal growth if eaten in sufficient amount." However, since the addition of animal protein to the diet greatly enhanced the value of the wheat protein for growth, a deficiency in certain amino acids was indicated. Csonka (5) has shown that wheat protein is very deficient in tryptophan. Other essential amino acids, notably lysine and methionine, are also present in rather small quantities (3).

Although studies relating the baking quality of wheat to chemical constitution have not been fruitful, certain relationships may become apparent with further work. The present study was initiated to obtain information on the effect of wheat species, variety, and environment on the cystine, methionine, lysine, and glutamic acid content of wheat protein.

### Materials and Methods

Three sets of wheat samples selected from pure species and varieties were obtained for this study. One set (Table I) consisted of several wheat species from the 1946 crop grown at Sacaton, Arizona. The second set of samples (Table I) representing hard red winter wheat were composited by variety from the 1947 crop for both the Central and Southern Great Plains. The Southern district samples were composited from equal portions of wheat grown at Amarillo, Chilli-



TABLE I  
WHEAT SAMPLES SELECTED FOR MICROBIOLOGICAL ASSAY  
OF THEIR CONSTITUENT AMINO ACIDS

| Name                     | Number       | %Protein <sup>1</sup> |                   |
|--------------------------|--------------|-----------------------|-------------------|
| WHEAT SPECIES            |              |                       |                   |
| <i>T. durum</i> (Pentad) | C. I. 3322   | 17.8                  |                   |
| <i>T. durum</i> (Mindum) | C. I. 5296   | 15.0                  |                   |
| <i>T. sphaerococcum</i>  | C. I. 4923   | 15.0                  |                   |
| <i>T. dicoccum</i>       | C. I. 7276   | 18.1                  |                   |
| <i>T. pyramidale</i>     | P. I. 113398 | 15.3                  |                   |
| <i>T. orientale</i>      | P. I. 68282  | 15.4                  |                   |
| <i>T. persicum</i>       | P. I. 115817 | 19.3                  |                   |
| <i>T. polonicum</i>      | P. I. 127087 | 18.9                  |                   |
| HARD RED WINTER WHEAT    |              |                       |                   |
| Kharkof                  | C. I. 1442   | 14.9 <sup>2</sup>     | 14.6 <sup>3</sup> |
| Blackhull                | C. I. 6251   | 14.9                  | 14.2              |
| Tenmarq                  | C. I. 6936   | 15.1                  | 13.4              |
| Pawnee                   | C. I. 11669  | 14.1                  | 14.5              |
| Comanche                 | C. I. 11673  | 14.9                  | 15.0              |
| Wichita                  | C. I. 11952  | 14.3                  | 14.3              |
| Red Chief                | C. I. 12109  | 14.2                  | 14.2              |

<sup>1</sup> Dry weight basis ( $N \times 5.7$ ).

<sup>2</sup> Samples from the Southern District.

<sup>3</sup> Samples from the Central District.

cothe, and Denton, Texas, and Lawton and Stillwater, Oklahoma. The Central district samples were composited from equal portions of wheat grown at Akron, Colorado, Manhattan, and Colby, Kansas, Alliance, Lincoln, and North Platte, Nebraska. The third set of samples (see Table IV) consisted of five varieties duplicated for the 1946 and 1947 crop years. These samples were composited by variety on the basis of similar protein content from many locations throughout the state of Kansas.

Samples were prepared for assay by hydrolyzing finely ground one-gram samples (dry weight basis, weighed to  $\pm 1$  mg.) in an autoclave at 15 lbs. pressure with 25 ml. of hydrochloric acid. The hydrolysis vessels were 125 ml. Erlenmeyer flasks covered with inverted beakers. Optimum conditions for hydrolysis varied for different amino acids as is graphically illustrated in Fig. 1. For both lysine and methionine assay, hydrolysis for 10 hours with 2 *N* hydrochloric acid was used. For cystine, optimum results were obtained by 1-hour hydrolysis with 4 *N* hydrochloric acid. Results obtained for cystine using 0.5 hour hydrolysis were low. (Not shown in Fig. 1.) For glutamic acid, maximum assay values were obtained by hydrolysis for five hours with 6 *N* hydrochloric acid. The hydrolysates were cooled, adjusted to pH 6.8<sup>2</sup> with sodium hydroxide solution, diluted to 100 ml. volume, and filtered.

<sup>2</sup> All pH adjustments were made using glass electrode equipment.

Microbiological methods of assay were employed throughout the study. These methods are suitable for obtaining comparative values and for analyzing a large number of samples at one time (Baumgarten *et al.*, 3). It is recognized that variable amounts of some amino acids, notably cystine (Riesen and coworkers, 18), are lost when heated in the presence of carbohydrates. To minimize this error all samples being compared were chosen for similarity of protein content and were hydrolyzed and analyzed simultaneously under similar conditions. The values reported, although not absolute, are nevertheless relative and adequate for indicating differences among samples.

The organisms used in this study were *Lactobacillus arabinosus* 17-5 and *Leuconostoc mesenteroides* P-60. These organisms were

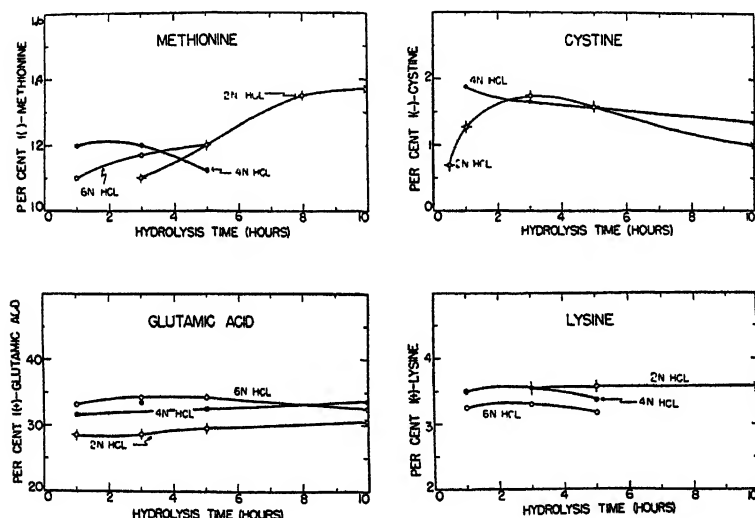


Fig. 1. Effect of hydrolysis time and acid strength on the rate of amino acid liberation from wheat protein.

obtained from the American type culture collection, Georgetown University School of Medicine, Washington, D. C.

The general procedure for carrying out the assays was that used by Kuiken and coworkers (13). The tubes, after sterilization, were cooled, inoculated, and incubated at 36°C. for 72 hours. Titrations were completed with 0.10 *N* sodium hydroxide in the 22 × 175 mm. incubation tubes without centrifuging. One ml. (0.7 mg.) of brom-thymol blue in 25% ethanol was used as the indicator. Typical standard curves are shown in Fig. 2.

Results from duplicate tubes and from all assay levels were averaged and reported as per cent of the total protein material present (dry weight basis).

The composition of the complete medium and the stock solutions used for the assay of glutamic acid with *Lactobacillus arabinosus* were essentially the same as those used by Riesen and coworkers (17).<sup>3</sup> The amounts of l-lysine and dl-threonine were decreased to 1 mg. per tube while 2 mg. of dl-tryptophan, 2 mg. of dl-glycine, 1 mg. of l-proline, and 1 mg. of dl-serine were added per assay tube. The quantities of purines and pyrimidine were doubled, while choline and inositol were omitted from the medium. As suggested by Lyman and

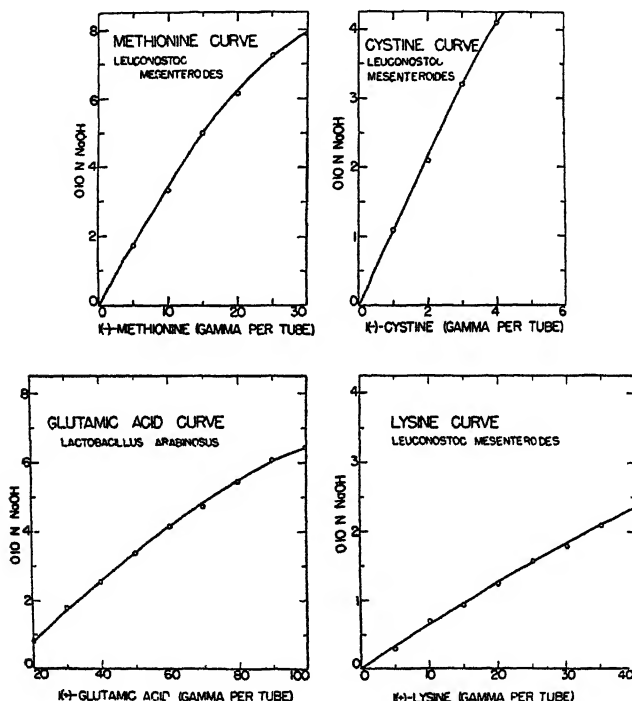


Fig. 2. Typical standard curves for the determination of methionine, cystine, glutamic acid, and lysine. Titration values are for the entire 10 ml. culture. The incubation time was 72 hours and the incubation temperature 36°C.

coworkers (14) glutamine was added to the medium in the proportion of 0.25 mg. per assay tube. Sigmoidal growth curves were obtained unless the glutamine was present in the medium.

The composition of the synthetic medium used for the assay of lysine with *Leuconostoc mesenteroides* was essentially the same as Medium C used by Dunn and coworkers (6). Hydroxyproline, norleucine, and norvaline were omitted from the medium.

<sup>3</sup> Certain changes in the basal medium for the assay of glutamic acid as well as for the other amino acids studied were made in order to obtain better growth curves under the conditions of the experiment and for purposes of standardization of the stock solutions.

The medium used for the microbiological assay of methionine using *Leuconostoc mesenteroides* as the test organism was a modification of that used for the determination of lysine. Hydrogen peroxide-treated peptone was prepared as described by Lyman *et al.* (15). The treated peptone solution was substituted for the pure amino acids with the exception of tryptophan, tyrosine, and cystine, which were added to the medium in the same quantities as used for the lysine analysis. Xanthine was included using 12 mg., per liter of basal medium.

*Leuconostoc mesenteroides* was selected as the most suitable organism for the microbiological assay of cystine (Riesen and coworkers, 18). The medium was the same as that used in the determination of methionine, with two exceptions. The amounts of glucose and sodium acetate used in the medium were halved. Cystine was omitted from the medium and methionine was added in the same quantity as used in the assay for lysine.

### Results and Discussion

The percentage of glutamic acid in several wheat species was determined to ascertain the desirability of continuing similar work on

TABLE II  
SUMMARY OF MICROBIOLOGICAL ASSAY DATA AND ANALYSES OF VARIANCE  
OF THE PER CENT GLUTAMIC ACID PRESENT IN THE TOTAL  
PROTEIN OF EIGHT WHEAT SPECIES

| Hydrolysis No. | Analysis No. | Per cent glutamic acid in protein ( $N \times 5.7$ ) on dry basis |                             |                         |                       |                      |                     |                    |                     | Average |
|----------------|--------------|-------------------------------------------------------------------|-----------------------------|-------------------------|-----------------------|----------------------|---------------------|--------------------|---------------------|---------|
|                |              | <i>T. durum</i><br>(Pentad)                                       | <i>T. durum</i><br>(Mildum) | <i>T. sphaerococcum</i> | <i>T. discococcum</i> | <i>T. pyramidale</i> | <i>T. orientale</i> | <i>T. persicum</i> | <i>T. polonicum</i> |         |
|                |              | %                                                                 | %                           | %                       | %                     | %                    | %                   | %                  | %                   | %       |
| 1              | 1            | 32.2                                                              | 30.4                        | 28.5                    | 33.1                  | 28.6                 | 31.9                | 31.0               | 32.2                |         |
| 2              | 1            | 37.4                                                              | 35.3                        | 28.9                    | 32.7                  | 29.7                 | 29.8                | 31.9               | 34.1                |         |
| 3              | 1            | 33.3                                                              | 31.6                        | 29.3                    | 33.4                  | 29.2                 | 30.9                | 32.6               | 33.3                |         |
| Average        |              | 34.3                                                              | 32.4                        | 28.9                    | 33.1                  | 29.2                 | 30.9                | 31.8               | 33.2                | 31.7    |
| 1              | 1            | 32.2                                                              | 30.4                        | 28.5                    | 33.1                  | 28.6                 | 31.9                | 31.0               | 32.2                |         |
| 1              | 2            | 35.7                                                              | 33.2                        | 29.8                    | 34.3                  | 28.7                 | 31.9                | 32.3               | 33.3                |         |
| 1              | 3            | 32.5                                                              | 32.6                        | 29.8                    | 32.7                  | 30.2                 | 32.6                | 32.5               | 35.0                |         |
| Average        |              | 33.5                                                              | 32.1                        | 29.4                    | 33.4                  | 29.2                 | 32.1                | 31.9               | 33.5                | 31.9    |

| ANALYSES OF VARIANCE                               |                    |              |
|----------------------------------------------------|--------------------|--------------|
| Source of variation                                | Degrees of freedom | Mean squares |
| Species                                            | 7                  | 11.307**     |
| Error (One analysis on each of three hydrolysates) | 16                 | 2.159        |
| Species                                            | 7                  | 9.057***     |
| Error (Three analyses on one hydrolysate)          | 16                 | 1.352        |

\*\* Significance exceeds the 1% level.

\*\*\* Significance exceeds the 0.1% level.

wheat varieties. A summary of assay data and the analysis of variance are shown in Table II. No significant differences were found between single analyses on each of three different hydrolysates and triple analyses on one hydrolysate. The differences in percentages of glutamic acid present in the wheat species tested were statistically

TABLE III

SUMMARY OF MICROBIOLOGICAL ASSAY DATA AND ANALYSES OF VARIANCE OF THE PER CENT GLUTAMIC ACID, METHIONINE, CYSTINE, AND LYSINE PRESENT IN THE TOTAL PROTEIN OF SEVEN COMPOSITED WHEAT VARIETIES GROWN IN TWO WIDELY DIFFERENT AREAS

| Variety                    | Protein <sup>1</sup> | Per cent of amino acid in protein ( <i>N</i> ×5.7) on dry basis <sup>2</sup> |              |           |          |
|----------------------------|----------------------|------------------------------------------------------------------------------|--------------|-----------|----------|
|                            |                      | 1-Glutamic acid                                                              | 1-Methionine | 1-Cystine | 1-Lysine |
| SOUTHERN DISTRICT          |                      |                                                                              |              |           |          |
|                            | %                    | %                                                                            | %            | %         | %        |
| Kharkof                    | 14.9                 | 32.5                                                                         | 1.37         | 2.10      | 3.40     |
| Blackhull                  | 14.9                 | 33.1                                                                         | 1.33         | 2.18      | 3.16     |
| Tenmarq                    | 15.1                 | 32.9                                                                         | 1.36         | 2.13      | 3.30     |
| Pawnee                     | 14.1                 | 32.0                                                                         | 1.43         | 2.19      | 3.18     |
| Comanche                   | 14.9                 | 33.5                                                                         | 1.41         | 2.19      | 3.12     |
| Wichita                    | 14.3                 | 33.3                                                                         | 1.37         | 2.14      | 3.16     |
| Red Chief                  | 14.2                 | 33.2                                                                         | 1.44         | 2.17      | 3.07     |
| Mean for Southern District | 14.6                 | 32.9                                                                         | 1.39         | 2.16      | 3.20     |
| CENTRAL DISTRICT           |                      |                                                                              |              |           |          |
|                            | 14.6                 | 33.3                                                                         | 1.43         | 2.26      | 3.39     |
| Kharkof                    | 14.2                 | 32.7                                                                         | 1.45         | 2.43      | 3.33     |
| Blackhull                  | 13.4                 | 33.5                                                                         | 1.54         | 2.47      | 3.41     |
| Tenmarq                    | 14.5                 | 32.8                                                                         | 1.46         | 2.37      | 3.21     |
| Pawnee                     | 15.0                 | 33.9                                                                         | 1.42         | 2.35      | 3.21     |
| Comanche                   | 14.3                 | 32.8                                                                         | 1.44         | 2.36      | 3.15     |
| Wichita                    | 14.2                 | 32.2                                                                         | 1.49         | 2.43      | 3.30     |
| Red Chief                  |                      |                                                                              |              |           |          |
| Mean for Central District  | 14.3                 | 33.0                                                                         | 1.46         | 2.38      | 3.29     |
| Mean for both districts    |                      | 33.0                                                                         | 1.43         | 2.27      | 3.25     |
| ANALYSES OF VARIANCE       |                      |                                                                              |              |           |          |
| Source of variation        | Degrees of freedom   | Mean square                                                                  |              |           |          |
|                            |                      | Glutamic acid                                                                | Methionine   | Cystine   | Lysine   |
| Variety                    | 6                    | 0.007                                                                        | 0.003        | 0.007     | 0.037    |
| District                   | 1                    | 0.001                                                                        | 0.040***     | 0.350***  | 0.056    |
| V×D                        | 6                    | 0.005                                                                        | 0.003        | 0.005     | 0.008    |
| Error                      | 14                   | 0.011                                                                        | 0.002        | 0.017     | 0.024    |

<sup>1</sup> Moisture-free basis.

<sup>2</sup> Mean for two separate analyses. Both hydrolysis and analyses of all varieties were performed simultaneously for each amino acid.

\*\*\* Significance exceeds the 0.1% level.

significant. It is possible that this is the result of differences in the relative proportions of the various protein types present in the wheat.

Based on the results obtained for glutamic acid in different wheat species, the work was extended to include the determination of four amino acids in seven hard red winter wheat varieties composited from samples grown in widely different areas. Data expressing the percentage of glutamic acid, methionine, cystine, and lysine present in the total wheat protein are recorded in Table III. A summary of the statistical analyses of these data is also shown in Table III. Differences in methionine, cystine, glutamic acid, and lysine among varieties were nonsignificant. Highly significant differences, however, were found for the methionine and the cystine content of varieties grown in different districts of the Great Plains area. The percentage of both sulfur-bearing amino acids in the wheat protein was consistently higher for all the varieties grown in the Central district. The similarity of the protein content for the samples obtained in the two districts (Table III) probably precludes the variation in the carbohydrate moiety as a factor in accounting for the differences found for the two districts.

It was thought that this difference among the samples from the two districts might be due to a greater quantity of available sulfur in the soils from the Central district. An analysis for sulfate in the soils obtained from the locations where the wheat samples were grown revealed that the average sulfate concentration per liter of saturation extract of the soils from the Southern and Central districts amounted to 0.0075 and 0.0178 milli-equivalents, respectively. Further studies involving the analysis for methionine in eleven samples of Comanche and Red Chief wheat grown in each of the eleven stations within the Southern and Central districts (1947 crop) revealed no significant correlation between per cent methionine and the sulfate concentration in soil samples obtained from the respective stations. It would appear, therefore, that environmental factors other than available soil sulfate resulted in the significant differences in methionine and cystine recorded in Table III. Differences in per cent methionine for the series of eleven samples representing Comanche and Red Chief wheat were significant for both station and variety. Further study of the factors governing this condition merits serious attention.

The assay values for cystine and the dough mixing time for five varieties of winter wheat grown during two crop years are summarized in Table IV. The wheat proteins contained significantly more cystine in 1947 than in 1946 and the flour from the 1947 samples also required longer mixing than did the corresponding flour samples from the 1946 crop. Thus, there may be a relationship between mixing time and

TABLE IV

SUMMARY OF MICROBIOLOGICAL ASSAY DATA, THE ANALYSIS OF VARIANCE OF THE PER CENT CYSTINE BASED ON TOTAL WHEAT PROTEIN, AND FLOUR MIXING TIME FOR FIVE VARIETY COMPOSITES OF WHEAT GROWN IN 1946 AND 1947

| Crop year                                              | Variety |                    |              |              |           | Average |
|--------------------------------------------------------|---------|--------------------|--------------|--------------|-----------|---------|
|                                                        | Tenmarq | Comanche           | E. Blackhull | Pawnee       | Red Chief |         |
| CYSTINE (PER CENT OF TOTAL WHEAT PROTEIN) <sup>1</sup> |         |                    |              |              |           |         |
| 1946                                                   | 2.42    | 2.43               | 2.49         | 2.42         | 2.48      | 2.45    |
| 1947                                                   | 2.66    | 2.49               | 2.69         | 2.55         | 2.80      | 2.64    |
| FLOUR MIXING TIME (MIN.)                               |         |                    |              |              |           |         |
| 1946                                                   | 2.3     | 2.8                | 1.4          | 1.2          | 2.2       | 2.0     |
| 1947                                                   | 3.0     | 2.8                | 1.6          | 2.3          | 2.6       | 2.5     |
| ANALYSIS OF VARIANCE FOR CYSTINE                       |         |                    |              |              |           |         |
| Source of variation                                    |         | Degrees of freedom |              | Mean squares |           |         |
| Variety                                                |         | 4                  |              | 0.033        |           |         |
| Year                                                   |         | 1                  |              | 0.273*       |           |         |
| V×Y                                                    |         | 4                  |              | 0.016        |           |         |
| Error                                                  |         | 20                 |              | 0.044        |           |         |

\* Significance exceeds the 5% level.

<sup>1</sup> Per cent cystine in protein ( $N \times 5.7$ ) on dry basis. Each value is the average of three separate analyses, each performed simultaneously for all varieties grown in both years.

TABLE V

COMPARISON OF MICROBIOLOGICAL VALUES WITH THOSE CITED IN THE LITERATURE<sup>1</sup>

| Amino acid    | Mean of microbiological values 'all varieties' <sup>2</sup> | Literature values                                                        |                                                           |
|---------------|-------------------------------------------------------------|--------------------------------------------------------------------------|-----------------------------------------------------------|
|               |                                                             | Microbiological values                                                   | Chemical values                                           |
| Cystine       | 2.27                                                        | 1.91 (1)                                                                 | 1.4±0.3 (4)<br>2.85 (7)                                   |
| Glutamic acid | 33.0                                                        | 35.75 (14)<br>32.4-26.4 (2) <sup>3</sup>                                 |                                                           |
| Lysine        | 3.25                                                        | 3.18 (19)<br>3.07 (1)<br>2.74 (12)<br>2.70 (3)                           | 2.91 (4)                                                  |
| Methionine    | 1.43                                                        | 1.32 (19)<br>1.45 (1)<br>2.81 (17)<br>1.28 (15)<br>1.08 (11)<br>1.35 (3) | 2.19 (4)<br>1.34 (11)<br>2.74 (7)<br>2.19 (8)<br>1.26 (5) |

<sup>1</sup> Expressed as per cent of the total protein ( $N \times 5.7$ ) on dry weight basis.

<sup>2</sup> Data from Table III.

<sup>3</sup> Depending on the organism employed.

per cent cystine (based on total wheat protein) as influenced by environment. The two samples of Comanche wheat which possessed the same optimum mixing time also contained the same percentage of cystine based on total wheat protein. The longer mixing varieties tended to reflect greater change in mixing requirement for a small change in cystine content. Further work relating cystine content and mixing requirement is in progress.

The reproducibility of the microbiological assay values is illustrated in Table II. The values obtained for all four amino acids are in line with chemical and microbiological assay values for wheat reported in the literature (Table V). The variations among reported values may in part be accounted for by sample variation. The present work indicates that the methionine and cystine content of wheat protein may vary significantly.

### Acknowledgments

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## CAKE PROPERTIES IN RELATION TO FLOUR PARTICLE SIZE FRACTIONS<sup>1</sup>

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### ABSTRACT

Patent, straight, and clear grade flours milled from an Ohio soft red winter wheat were fractionated into three particle size groups measured by diameters of  $37\mu$  or less, over  $37\mu$  up to and including  $53\mu$ , and larger than  $53\mu$ . Differences in granulation of the three flour grades were minor, but the quantity of coarse material increased as the flour grade decreased.

The properties of the different particle size fractions from each grade of flour varied widely. The 0- $37\mu$  fractions for each flour were lowest in protein, ash, and viscosity and produced the best cakes. The fractions containing the largest sized particles were intermediate in protein and ash content, had the highest viscosity, and produced the poorest cakes. The 37- $53\mu$  fractions were highest in protein and ash content, and intermediate in viscosity and cake baking quality.

Wichser and Shellenberger (6, 7, 8) have reported previously on comparative granulation studies of flour produced from three classes of wheat. Various chemical and physical tests, including baking response, were reported for the different particle size fractions. However, only one soft wheat was tested and the cookie test was the only baking test applied. The present study expands the flour granulation research by including cake baking tests of several flour fractions from three grades of soft wheat flour.

### Materials and Methods

Patent, straight, and clear grade cake flours were milled from the same Ohio soft red winter wheat. All samples were bleached with

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TABLE I  
ANALYSIS OF SOFT WHEAT FLOUR PARTICLE SIZE FRACTIONS<sup>1</sup>

| Flour grade  | Flour fraction size <sup>2</sup> | Protein  | Ash      | pH  | Viscosity    |
|--------------|----------------------------------|----------|----------|-----|--------------|
|              | <i>microns</i>                   | <i>%</i> | <i>%</i> |     | <i>MacM.</i> |
| Short patent | Control                          | 7.5      | 0.33     | 5.0 | 28           |
|              | 0-37                             | 6.5      | .32      | 5.0 | 19           |
|              | 37-53                            | 9.1      | .37      | 5.0 | 78           |
|              | >53                              | 8.7      | .33      | 5.0 | 81           |
| Straight     | Control                          | 7.6      | .39      | 5.1 | 25           |
|              | 0-37                             | 6.7      | .37      | 5.1 | 17           |
|              | 37-53                            | 9.2      | .46      | 5.1 | 60           |
|              | >53                              | 9.1      | .40      | 5.1 | 83           |
| Clear        | Control                          | 7.7      | .49      | 5.2 | 20           |
|              | 0-37                             | 6.9      | .46      | 5.2 | 13           |
|              | 37-53                            | 9.2      | .57      | 5.2 | 45           |
|              | >53                              | 9.0      | .52      | 5.2 | 95           |

<sup>1</sup> Results reported on 14% moisture basis.

<sup>2</sup> 0-37 $\mu$  inclusive, over 37 $\mu$  up to and including 53 $\mu$ , and over 53 $\mu$ .

a commercial preparation containing benzoyl peroxide and chlorinated. The pH values are shown in Table I.

The flours were separated into three fractions based on particle size using Tyler wire screens and a Ro-Tap shaker as described by Wichser

TABLE II  
CAKE BAKING FORMULA AND MIXING PROCEDURES

| Ingredients            | Percentage based on flour | Mixing procedure <sup>1</sup>                                                                                                                                                                                                                                                                         |
|------------------------|---------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|                        | <i>%</i>                  |                                                                                                                                                                                                                                                                                                       |
| Flour                  | 60.0                      | { Mix for 1.5 min. at 62 rpm. Mix for 1 min. additional time at 128 rpm. Scrape down and continue to mix for ½ min. and again scrape down.                                                                                                                                                            |
| Emulsified shortening  | 40.0                      |                                                                                                                                                                                                                                                                                                       |
| Water                  | 45.0                      | { Add all the water. Then add the blended and sifted dry material. Mix for 2 min. at 62 rpm. Scrape down. Continue mixing at same speed for 1 additional minute.                                                                                                                                      |
| Sugar (sucrose)        | 125.0                     |                                                                                                                                                                                                                                                                                                       |
| Flour                  | 40.0                      |                                                                                                                                                                                                                                                                                                       |
| Salt                   | 2.5                       |                                                                                                                                                                                                                                                                                                       |
| Baking powder          | 5.5                       |                                                                                                                                                                                                                                                                                                       |
| Milk solids (fat-free) | 15.0                      | { Mix egg whites and water, adding one-half of the mixture gradually during a period of 1 min. at 62 rpm. Scrape down. Mix an additional minute at same speed. Add balance of liquid gradually during 1 min. at 62 rpm. Scrape down. Mix 3 min. at 62 rpm., then finish mixing at 128 rpm. for ½ min. |
| Phosphate (V-90)       | 0.5                       |                                                                                                                                                                                                                                                                                                       |
| Egg white              | 60.0                      | { Mix egg whites and water, adding one-half of the mixture gradually during a period of 1 min. at 62 rpm. Scrape down. Mix an additional minute at same speed. Add balance of liquid gradually during 1 min. at 62 rpm. Scrape down. Mix 3 min. at 62 rpm., then finish mixing at 128 rpm. for ½ min. |
| Water                  | 40.0                      |                                                                                                                                                                                                                                                                                                       |

<sup>1</sup> Kitchen Aid Model G mixer equipped with 4-quart bowl was used.

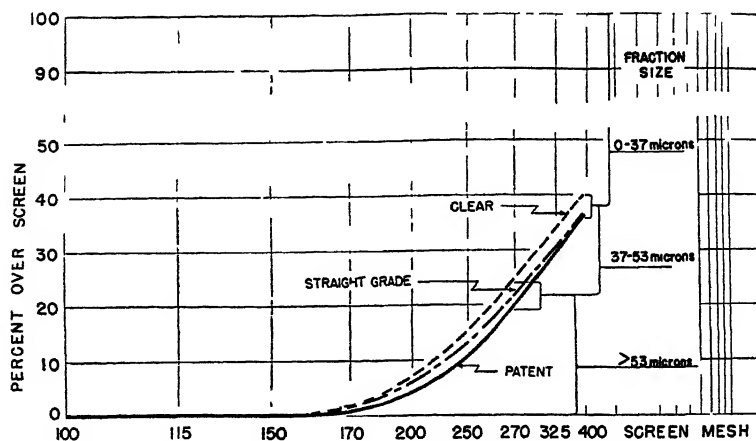


Fig. 1 Granulation curves of patent straight, and clear grade, soft wheat flours (0-37 $\mu$  inclusive, over 37 $\mu$  up to and including 53 $\mu$ , and over 53 $\mu$ .)

and Shellenberger (6, 7, 8). The analyses of the original flours and the flour fractions are shown in Table I.

Viscosity determinations were made on acidulated flour-water

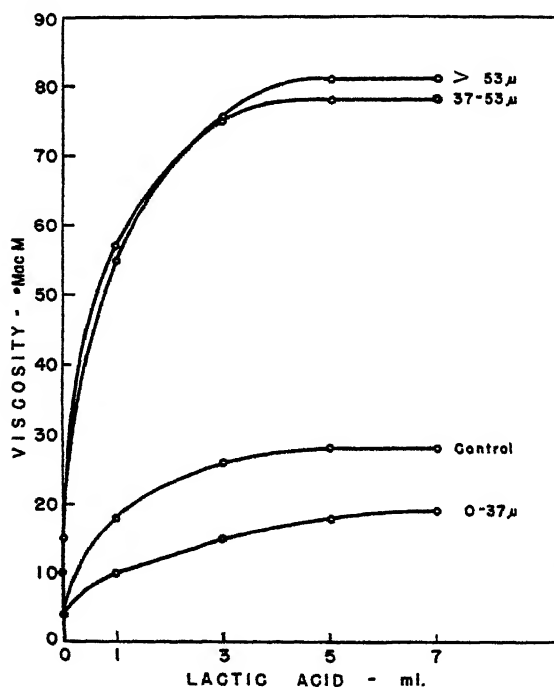


Fig. 2. Viscosity curves of particle size fractions from patent cake flour. (0-37 $\mu$  inclusive, over 37 $\mu$  up to and including 53 $\mu$ , and over 53 $\mu$ .)

suspensions using the MacMichael viscosimeter as described in Cereal Laboratory Methods, 5th ed. (1).

Cake baking tests were conducted using a commercial white cake formula. The ingredients and the mixing procedure are recorded in Table II. The batters (400 g.) were placed in round,  $8 \times 1.5$  in. pans, and baked for 25 min. at  $375^{\circ}\text{F}$ .

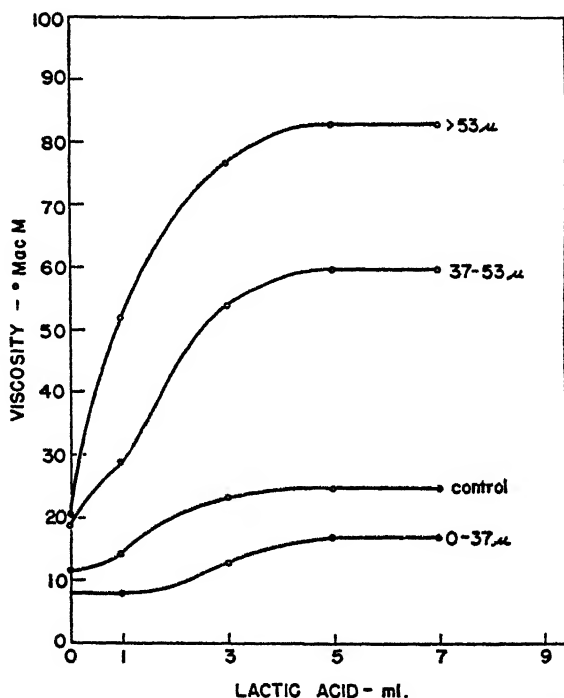


Fig. 3. Viscosity curves of particle size fractions from straight grade cake flour. (0-37 $\mu$  inclusive, over 37 $\mu$  up to and including 53 $\mu$ , and over 53 $\mu$ .)

## Results

*Granulation of Cake Flours.* The granulation curves for the three flours are shown in Fig. 1, using the cumulative direct plot diagram (6). The proximity of the curves indicates that the differences in granulation were slight between the patent, straight, and clear grade flours milled from the same wheat blend. All three flours were finely ground, compared with hard wheat flours, as is shown by the fact that less than 40% of the flour particles fail to pass through a No. 400 Tyler wire screen. Approximately 70% of the usual hard wheat flour fails to pass through a No. 400 screen.

Although the granulation curves for the three flours are similar, the

curves show that for each particle size fraction the amount of coarser material increases as the flour grade decreases.

*Ash and Protein in Cake Flour Fractions.* The same relationship between particle size, ash, and protein exists as has been reported previously for soft wheat flour (6, 7). The smallest particle size group ( $0-37\mu$ ) is lowest in ash and protein. The intermediate particle size fraction ( $37-53\mu$ ) is highest in both ash and protein. The flour fraction containing particles larger than  $53\mu$  is intermediate between the other two groups in ash and protein content. In contrast to the results ob-

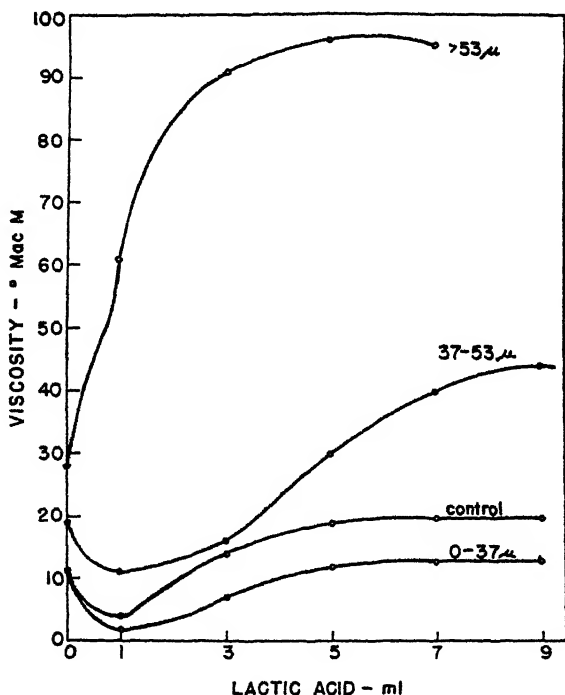


Fig. 4. Viscosity curves of particle size fractions from clear grade cake flour. ( $0-37\mu$  inclusive, over  $37\mu$  up to and including  $53\mu$ , and over  $53\mu$ .)

tained with hard wheat flour (8) in which the small particle size fraction ( $0-37\mu$ ) was low in protein but high in ash, the soft wheat flour fractions show a direct relationship throughout between ash and protein. Fractions highest in protein were found to be highest in ash.

*Viscosity of Cake Flour Fractions.* The relationship between MacMichael viscosity and flour particle size for the three flour types is illustrated graphically in Figs. 2, 3, and 4. Viscosity increased greatly with increase in flour particle size. In every case the smaller particle size fraction ( $0-37\mu$ ) had the lowest viscosity while the largest particle

size fraction had the highest viscosity. Since approximately 60% of the flour consisted of the 0-37 $\mu$  fraction, it would be expected that the viscosity of the original flour (control) would be similar to the curve for the smallest size particle group. The expected greater buffering effect (3) of higher ash flour is apparent in the clear flour measurements.

There was an apparent relationship between viscosity readings and the quality of the cakes produced from the different flour fractions. With one exception the low viscosity flour fractions produced the best cakes.

**Cake Baking Tests.** The results of the baking tests are recorded in Table III and photographs of the cakes are shown in Figs. 5, 6, and 7. For all three flour grades the fractions containing particles within

TABLE III  
THE EFFECT OF FLOUR PARTICLE SIZE ON THE QUALITY OF CAKES

| Flour grade | Flour fractions <sup>1</sup> | Cake grading characteristics |                         |            |                   |             |      |
|-------------|------------------------------|------------------------------|-------------------------|------------|-------------------|-------------|------|
|             |                              | Volume                       | Vol. index <sup>2</sup> | Appearance | Grain and texture | Crumb color | Rank |
| Patent      | <i>microns</i>               | <i>ml.</i>                   | <i>in.</i>              |            |                   |             |      |
|             | Control                      | 980                          | 2 7/8                   | 4          | 4                 | Good        | 4    |
|             | 0-37                         | 1100                         | 3 5/16                  | 1          | 1                 | Bright      | 1    |
|             | 37-53                        | 1050                         | 3 1/8                   | 2          | 2                 | Gray        | 2    |
|             | >53                          | 1000                         | 3                       | 3          | 3                 | Sl. gray    | 3    |
| Straight    | Control                      | 1005                         | 3                       | 2          | 2                 | Bright      | 2    |
|             | 0-37                         | 1070                         | 3 1/4                   | 1          | 1                 | Bright      | 1    |
|             | 37-53                        | 1040                         | 3 1/8                   | 3          | 3                 | Dull        | 3    |
|             | >53                          | 983                          | 3                       | 4          | 4                 | Dull        | 4    |
|             |                              |                              |                         |            |                   |             |      |
| Clear       | Control                      | 985                          | 2 11/16                 | 4          | 2                 | Sl. dull    | 2    |
|             | 0-37                         | 1108                         | 3 1/4                   | 1          | 1                 | Sl. bright  | 1    |
|             | 37-53                        | 1030                         | 3                       | 2          | 3                 | Sl. gray    | 3    |
|             | >53                          | 1013                         | 2 13/16                 | 3          | 4                 | Gray        | 4    |
|             |                              |                              |                         |            |                   |             |      |

<sup>1</sup> 0-37 $\mu$  inclusive, over 37 $\mu$  up to and including 53 $\mu$ , and over 53 $\mu$ .

<sup>2</sup> Volume index obtained by slicing layer in half and placing the two halves one on top of the other. The measurement is the thickness at the center of the two half slices of cake. A top quality cake should measure 3 1/4 in. in height.

the size range of 0-37 $\mu$  produced the best cakes and were ranked No. 1. It is evident in these studies that cake quality improved with a decrease in particle size. This corroborates the work of Schreck and Gerrits (5) and Alexander (2). The only exception to this observation was the patent grade for which the unfractionated flour produced less satisfactory cakes than did any of the fractions from the same sample. Since all particle size groups of the short patent flour produce good cakes, uniformity of particle size may be more important than particle size. Kress (3) has stated that for bread baking purposes the more uniform the granulation the better the baking quality, and Patterson

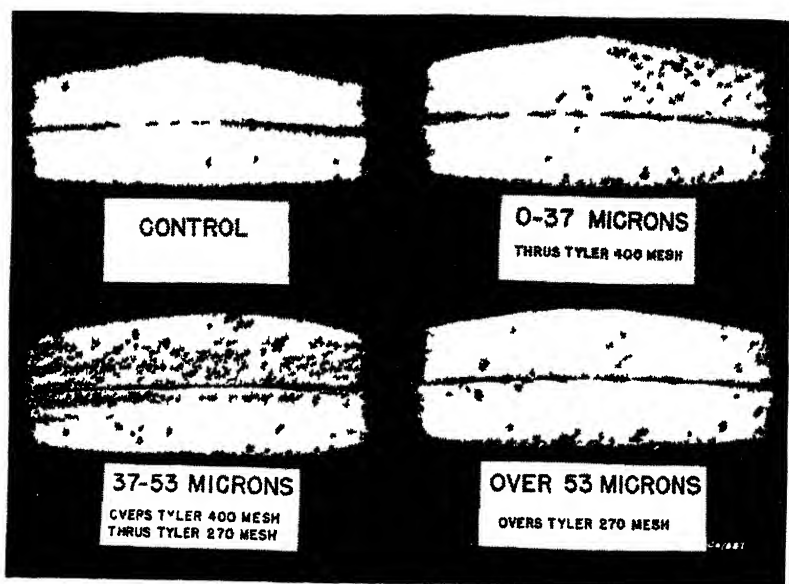


Fig 5 Cakes made from patent grade flour fractions (0-37 $\mu$  inclusive over 37 $\mu$  up to and including 53 $\mu$  and over 53 $\mu$ )

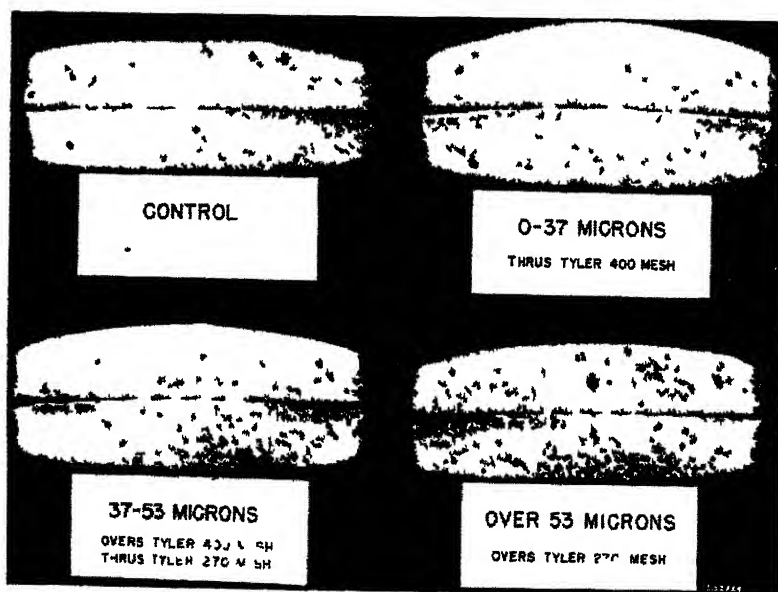


Fig 6 Cakes made from straight grade flour fractions (0-37 $\mu$  inclusive, over 37 $\mu$  up to and including 53 $\mu$ , and over 53 $\mu$ )

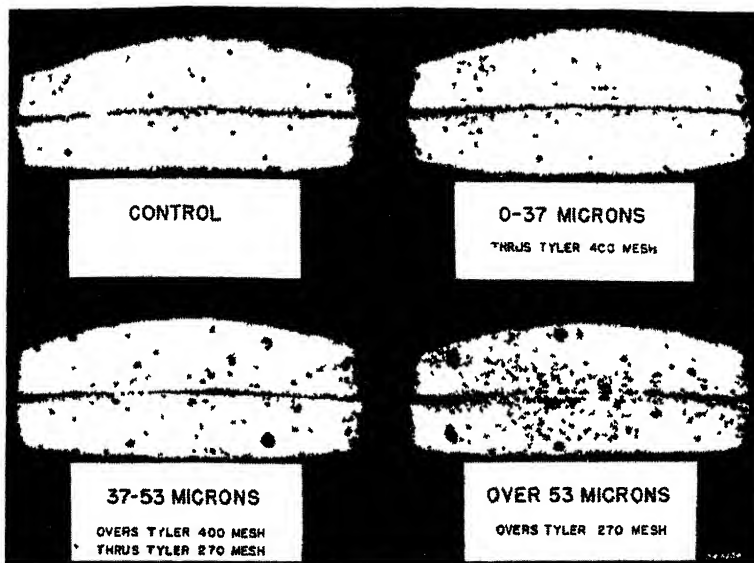


Fig 7 Cakes made from clear grade flour fractions. (0-37 $\mu$  inclusive, over 37 $\mu$  up to and including 53 $\mu$ , and over 53 $\mu$ )

(4) has indicated that the cake-making qualities of a flour were dependent on uniformly fine division of the flour particles.

The studies dealing with three grades of soft red winter wheat flour showed that the cake baking properties of the flour fractions improved with a decrease in particle size and a decrease in viscosity.

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# THE *IN VITRO* DIGESTIBILITY OF RAW AND HEAT-PROCESSED SOY PRODUCTS VARYING IN THE NUTRITIVE VALUE OF THE PROTEIN<sup>1</sup>

MORRIS SIMON<sup>2</sup> and DANIEL MELNICK<sup>3</sup>

## ABSTRACT

The susceptibility of the protein in commercially available soy products to *in vitro* enzymic digestion has been compared with the nutritional value obtained by animal assay methods, using soy products in various stages of heat-processing. These consisted of: (1) dehulled soybeans, (2) unheated, defatted soy flakes, (3) mildly heat-processed soy flour, (4) soy flour, optimally heat-processed with respect to the nutritional quality of the protein, and (5) an over-heat-processed flour experimentally produced for the present study.

The presence in raw soy products of a naturally-occurring proteolytic factor interfered with the *in vitro* digestion technique by acting synergistically with the pancreatin employed in the test. However, poor correlation was obtained between the nutritive data and the *in vitro* digestibility values for the protein in the soy flour samples, even when the interfering factor was inactivated. Large increases in the efficiency with which the protein in the soy products was utilized by the test animals were associated with only small increases in *in vitro* digestibility. However, in the case of the sample which had been purposely overheated, a large increase in the susceptibility of the protein to *in vitro* digestibility has been noted, and this is associated with only a small decline from the optimum nutritive value.

The nutritive value of the protein in soy products can be markedly increased without appreciably affecting antitryptic activity. The findings supplement but do not contradict earlier reports in the literature pointing to an inverse correlation between the protein value of soy products and antitryptic potency.

The improvement in nutritive value of soybean protein, effected by heat treatment in the presence of water, has been demonstrated by numerous investigators in laboratory feeding experiments with chicks (18), mice (33), and rats (20). Various suggestions have been offered to explain this increase in the biological value of the protein. A substance capable of inhibiting the activity of trypsin was found in raw soybean oil meal by Ham and Sandstedt (15) and by Bowman (8). This substance was later crystallized by Kunitz (22) and shown to be a

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protein. The naturally-occurring trypsin inhibitor has been held to be largely responsible for the interference in the biological utilization of the protein. Addition of the inhibitor to otherwise adequate diets was shown to impair the growth rate of chicks (16) and of rats (20). That the inhibitor is destroyed or rendered inactive by heat has been demonstrated in both *in vivo* (20, 32, 33) and *in vitro* (7, 33) experiments.

It was emphasized by Melnick and associates (27) that the increase in nutritive value of the protein resulting from heat treatment was accompanied by an increase in the susceptibility of the protein to *in vitro* pancreatic digestion. In support of their findings, Evans (12, 14), using trypsin or trypsin and erepsin, Jones (19), using trypsin, and Riesen *et al.* (30), using pancreatin, observed that a marked increase in *in vitro* digestibility occurred when soybean oil meal was autoclaved or cooked in water. Harte (17), using egg white, in which the existence of a trypsin inhibitor has been conclusively demonstrated (2, 24), found that the heat-coagulated material was rapidly digested *in vitro* by pancreatin while the unheated material showed no significant digestion even after 24 hours. In casein, however, where heating was known to impair the nutritive value of the protein, its susceptibility to *in vitro* enzymic digestion was found to be concomitantly decreased (4, 29).

The present investigation is part of an over-all study, undertaken to characterize soy products used as such or as ingredients in food formulations for human feeding. The paper (25) preceding this one is concerned with an evaluation of the functional (utility) properties of the protein in commercially available soy products, with special emphasis on curd-producing capacity. The current study was designed to determine the relationship between the nutritive value and *in vitro* digestibility of the protein, and to determine therefrom the suitability of the *in vitro* digestion technique as an objective test procedure for characterizing soy products with regard to nutritive properties.

### Materials and Methods

The soybean flours employed in this study were all prepared from the same batch of soybeans and were representative of raw and heat-treated commercial products. These samples were furnished by the Soya Food Research Council, Soy Flour Association. The products consisted of: (1) dehulled soybeans, (2) unheated, solvent-extracted soy flakes, (3) bakery-type soy flour, mildly heated in order to eliminate the bitter qualities of the raw bean but not sufficiently heated to seriously impair its functional properties, (4) a product optimally heat-processed with respect to the nutritive value of the protein, and (5)

an overheated product experimentally produced for the present study. Samples 2, 3, 4, and 5, commercially solvent-extracted, were ground and sifted through a No. 100 U. S. Standard sieve. Sample 1, the dehulled soybeans, was first ground through a sieve with openings 1 mm. in diameter, defatted with Skellysolve B, and finally ground to pass a No. 100 sieve.

Special equipment was employed in the commercial preparation of sample 2, in order to keep at a minimum the heat involved in solvent extraction of the oil and subsequent removal of the solvent from the product. In the case of sample 3, the bakery-type soy flour, the amount

TABLE I  
INFLUENCE OF HEAT-PROCESSING ON THE PROPERTIES OF COMMERCIALY  
PRODUCED SOYBEAN PRODUCTS<sup>1</sup>

| Sample/number and description                            | 1.<br>Unextracted<br>dehulled<br>soybeans | 2.<br>Solvent<br>extracted,<br>unheated | 3.<br>Solvent<br>extracted,<br>bakery<br>type | 4.<br>Solvent<br>extracted,<br>optimally<br>heated | 5.<br>Solvent<br>extracted,<br>overheated |
|----------------------------------------------------------|-------------------------------------------|-----------------------------------------|-----------------------------------------------|----------------------------------------------------|-------------------------------------------|
| Moisture, %                                              | 6.1                                       | 7.3                                     | 7.2                                           | 6.4                                                | 4.2                                       |
| Fat, %                                                   | 21.3                                      | 0.9                                     | 0.6                                           | 0.6                                                | 0.4                                       |
| Total protein, %                                         | 41.9                                      | 52.1                                    | 53.6                                          | 54.1                                               | 55.5                                      |
| Water soluble protein,<br>% of total                     | 76.7                                      | 83.1                                    | 65.5                                          | 22.5                                               | 6.2                                       |
| $\beta$ -amylase <sup>2</sup> ( $^{\circ}$ Lintner)      | 110.0                                     | 135.0                                   | 5.4                                           | 0.9                                                | 2.7                                       |
| Urease activity <sup>3</sup> (in-<br>crease in pH)       | 1.75                                      | 1.90                                    | 1.70                                          | 0.2                                                | 0.05                                      |
| Protein efficiency (g.<br>gained/g. protein<br>consumed) | 0.65                                      | 0.79                                    | 1.52                                          | 1.96                                               | 1.78                                      |

<sup>1</sup> Samples and data were supplied by Dr. J. W. Hayward, Archer-Daniels-Midland Co. Minneapolis, Minnesota, acting in behalf of the Soya Food Research Council, Soy Flour Association. All samples were from the same batch of soybeans.

<sup>2</sup> Modified procedure of the A.O.A.C., 6th ed. (1945). The  $\beta$ -amylase activity is determined by its hydrolytic action on starch. The maltose produced is measured by the reduction of a standard solution of potassium ferricyanide.

<sup>3</sup> Procedure of Caskey, C. D., and Knapp, F. C., Ind. Eng. Chem., Anal. Ed. 16: 640 (1944). The increase in pH is due to the liberation of ammonia from urea by the action of urease.

of heat used was the least necessary, within the limitations of conventional equipment, to eliminate the bitter qualities of the material.

The data presented in Table I are indicative of the degree of heat treatment to which the samples were exposed during commercial processing. The insignificance of the heat involved in the preparation of sample 2, the raw product, is evidenced by the retention of all its heat-labile properties. The mild heat treatment accorded sample 3, the bakery-type product, is manifested by its having suffered only a slight loss in urease activity and in water solubility of the protein. Caskey and Knapp (9) and Bird *et al.* (3) concluded from the results of their studies of the effect of heat on the urease activity and nutritive

value of the soybean protein that an increase in pH of 1.0 or more in the urease test employed by them indicated that the soybean meal had been inadequately heated to attain optimum nutritive value of the protein.  $\beta$ -amylase, on the other hand, because of its extreme heat-lability, is rapidly inactivated even at relatively low temperatures (10), and cannot adequately be employed to distinguish products that have been heated mildly from those which have been more severely heated. It will be observed that the protein solubility of the optimally heated product has been considerably reduced, while that of the excessively heated product has been almost entirely eliminated. The urease and  $\beta$ -amylase activity of both products are negligible.

*In Vitro Digestibility.* The susceptibility of the protein to *in vitro* enzymic digestion was determined according to the procedure of Melnick and associates (27, 28). A sample containing 6 gm. of protein ( $N \times 6.25$ ) was suspended in 50–60 ml. of water and the pH adjusted to 8.4. Thirty ml. of 0.075 *M* phosphate buffer solution<sup>4</sup> at the same pH were then added to the suspension and the volume brought to 130 ml. with water. Following the addition of 20 ml. of the buffer solution containing 200 mg. of U.S.P. pancreatin,<sup>5</sup> the suspension was thoroughly mixed, covered with 10 ml. of toluene, and incubated at 37°C. Five ml. aliquots of the enzymic digests were withdrawn at fixed intervals and the degree of hydrolysis determined by the increase in formol titratable nitrogen. The titration values for the undigested materials were determined on aliquots of similarly prepared samples to which heat-inactivated pancreatin had been added. The maximum formol titration values were obtained on acid hydrolysates of the samples.<sup>6</sup> The degree of hydrolysis at a given interval was expressed as the increase in the formol titratable nitrogen at that interval, divided by the maximum increase in this value (i.e., the figure for the acid hydrolyzed sample). The value thus obtained multiplied by 100 gave the per cent hydrolysis.

*Antitryptic Activity.* The inhibitor potency of the soy products was determined by a modification of the procedure described by Westfall and Hauge (33). This method involves the measurement of the ability of the anti-tryptic factor contained in the soy flour to retard the *in vitro* pancreatic digestion of casein. Twenty ml. of 0.5% pancreatin suspension buffered at pH 8.3 were pipetted into a glass-stoppered Erlenmeyer flask which was then placed in a 37°C. water bath. Five ml. of 0.5% buffered flour suspension were then added to the pancreatin, followed in exactly 5 minutes by 25 ml. of 4% sodium

<sup>4</sup> 750 ml. of *M*/5  $\text{KH}_2\text{PO}_4$  plus 144 ml. of 1 *M* NaOH diluted to 1,000 ml.: pH 8.4.

<sup>5</sup> Purchased from Central Scientific Co., Chicago, Ill.

<sup>6</sup> A sample containing 6 gm. of protein was refluxed for 24 hours with 8 *N*  $\text{H}_2\text{SO}_4$ . The hydrolysate was cooled, neutralized, and diluted to 150 ml. Aliquots were withdrawn, buffer added to the same concentration as in aliquots of the enzymic digests, and assayed by formol titration.

caseinate solution similarly buffered. Aliquots were withdrawn at specific intervals and the liberated amino nitrogen was measured by the formol titration technique. Corrections were made for the initial formol titratable nitrogen and that contributed by the inhibitor carrier, in this case the soy flour. The increase in formol titration was plotted against time of digestion and the relative velocity constant calculated from the reciprocal of the time required to decompose a fixed quantity of substrate. Inhibition was expressed as the per cent decrease from the relative velocity constant obtained when casein digestion was unretarded. Details of the procedure will be published at a later date.

*Protein Efficiency.* The nutritional data presented here were supplied by Mr. Ralph Holder of the Central Soya Company, Decatur, Indiana. Diets containing the soy products as the source of protein were fed *ad libitum* to duplicate groups of five weanling male albino rats of the Sprague-Dawley strain for a period of eight weeks during which food consumption and body weight changes were recorded at regular intervals. The ration furnished approximately 11% protein ( $N \times 6.25$ ). Protein efficiency was expressed as the grams of weight gained per gram of protein consumed. In addition to the protein contributing component, the composition of the basal diet was as follows: 10% sucrose; 3% ruffex; 4% salt mixture (Wesson Modification of the Osborne-Mendel salt mixture) (31); 2% 400-D/2,000-A oil, with 1.25 mg.  $\alpha$ -tocopherol added per g. of oil; 1% condensed fish solubles; 2% vitamin mixture; an amount of soybean oil sufficient to make 10% when added to the oil present in the test sample; corn starch to make a total of 100%. The vitamin mixture supplied per 100 g. of feed: 400  $\mu$ g. thiamine; 400  $\mu$ g. riboflavin; 400  $\mu$ g. pyridoxine; 3.5 mg. niacin; 1.1 mg. calcium pantothenate; 7.5 mg. para-amino benzoic acid; 30 mg. inositol; 200 mg. choline chloride; and 400  $\mu$ g. menadione. 1% of condensed fish solubles (50% solids) were used to supply the unidentified growth factors.

### Results and Discussion

The results obtained in the digestibility and anti-tryptic activity tests are presented in Table II, together with the nutritional values (efficiency of protein utilization) for the five samples. It will be noted that the mild heat treatment employed in processing the bakery-type flour has produced no measurable loss in its *in vitro* anti-tryptic activity. The nutritional value, on the other hand, has been considerably improved. In the optimally heated sample, the large increase in protein efficiency was accompanied by a marked decrease in anti-tryptic activity. However, in the case of the so-called "overheated"

sample, where the anti-tryptic activity has been still further reduced, efficiency of protein utilization has not been improved; actually, a slight decline from the optimum resulted. The impairment in biological value of the protein due to excessive heating has been attributed to a loss in the biological availability of lysine (5, 11, 26, 29) and methionine (13, 21). In their studies of the heat destruction of the soybean inhibitor as related to the heat destruction of urease, Borchers *et al.* (7) observed that the inhibitor survived longer periods of heating, at atmospheric and elevated pressures, than did urease. They re-

TABLE II

THE *IN VITRO* DIGESTIBILITY, ANTI-PROTEOLYTIC ACTIVITY, AND NUTRITIVE VALUE OF COMMERCIALY AVAILABLE SOYBEAN FLOURS IN VARIOUS STAGES OF HEAT-PROCESSING

| Sample                      | Extent of hydrolysis <sup>1</sup> |              |              | Anti-proteolytic activity <sup>2</sup><br>(per cent inhibition) | Protein efficiency <sup>3</sup>               |
|-----------------------------|-----------------------------------|--------------|--------------|-----------------------------------------------------------------|-----------------------------------------------|
|                             | After 1 day                       | After 2 days | After 5 days |                                                                 |                                               |
| 1<br>Raw flour              | %<br>12                           | %<br>16      | %<br>22      | 57                                                              | <i>gms. gained/<br/>gms. consumed</i><br>0.65 |
| 2<br>Raw flour              | 12                                | 15           | 20           | 57                                                              | 0.79                                          |
| 3<br>Bakery-type flour      | 6                                 | 9            | 15           | 57                                                              | 1.52                                          |
| 4<br>Optimally heated flour | 13                                | 19           | 23           | 33                                                              | 1.96                                          |
| 5<br>Overheated flour       | 16                                | 20           | 29           | 15                                                              | 1.78                                          |

<sup>1</sup> Based upon the ability of the proteolytic enzymes in the test system, containing a suboptimal quantity of added pancreatin, to digest the soybean protein.

<sup>2</sup> Based upon the ability of the soybean inhibitor to retard the *in vitro* pancreatic digestion of casein.

<sup>3</sup> Eight weeks' rat growth assays conducted by Mr. Ralph Holder of the Central Soya Co., Decatur, Indiana; the ration furnished approximately 11% protein ( $N \times 6.25$ ).

ported that the urease test could not be employed as a reliable index for the adequacy of heat treatment for the destruction of the inhibitor.

Poor correlation has been obtained between the protein efficiency data and the *in vitro* digestibility values for the protein in the soy flour samples. Large increases in the efficiency with which the soy products are used by the test animals are not associated with an increase but with an apparent loss of susceptibility to *in vitro* enzymic digestion in the case of sample 3, the mildly heat treated product, and with only a slight improvement in the digestibility of sample 4, the optimally heated product. However, in the case of sample 5, which had been purposely overheated, a large increase over the value obtained for the

raw material has been effected in the susceptibility of the protein to *in vitro* enzymic digestion, and this is associated with only a small decline from the optimum nutritive value. The present findings are not considered to be inconsistent with reports in the literature where large differences between the biological value of the protein in raw soy products and that in the same product heated to presumably optimum value have shown good correlation with the results yielded by *in vitro* digestibility studies. In very few, if any, of the studies reported in the literature have both animal assays and *in vitro* digestibility tests been conducted on the same sample in various stages of heat-processing. In most cases, comparisons were made only between the raw product and the same material heated to what was presumed to be optimum nutritive value. In the present study, the protein in the excessively heated product has been found to exhibit considerable improvement (more than 100%) in biological value over that of the raw product, and also a large increase in susceptibility to *in vitro* enzymic digestion.

The impairment in the susceptibility of the soy protein to *in vitro* enzymic digestion resulting from mild heat treatment is explained by the existence in raw soy products of naturally-occurring proteolytic enzymes. The occurrence of such proteases was reported by Melnick and Oser (28) who pointed out that the proteolysis due to these enzymes and that due to the added pancreatin was synergistic rather than additive, each factor augmenting the action of the other so that the net effect is greater than the sum of the separate effects. The presence of proteases in raw soy products is indicated by the data in Table III. Incubation of the raw product without pancreatin resulted in an increase in formol-titratable nitrogen. This increase was reduced to a trace when an aqueous suspension of the material was heated for 30 minutes at 60°C. prior to incubation without pancreatin. At 80°C. no measurable increase was observed. Concurrent with the loss in activity of the soybean protease, there occurred a decrease in the *in vitro* digestibility of the protein by pancreatin, the extent of protein digestion being less than that calculated by subtracting the results obtained in the test system containing only active soy proteases from that containing the added pancreatin. The absence of any increase in formol-titratable nitrogen in the bakery-type product, when incubated without pancreatin, indicates that the naturally-occurring proteolytic factor had been inactivated by the heat-processing employed in its manufacture commercially. Consequently, no impairment in its *in vitro* digestibility by pancreatin was effected by heating its aqueous suspension at 60°C. as in the case of the raw product. However, heating the aqueous suspension at temperatures exceeding 60°C. resulted in improvement of the *in vitro* digestibility. This

improvement may be attributed to the effect of the additional heat treatment in increasing the denaturation of the protein or upon the destruction of the soybean inhibitor or to a combination of both. The greater *in vitro* digestibility of the protein or the raw product when heated (in aqueous suspension) at 80°C. and 95°C. than that of the same product heated at 60°C. may be explained in the same manner. The higher temperatures were capable of inactivating the soybean

TABLE III  
INFLUENCE OF NATURALLY-OCCURRING PROTEOLYTIC ENZYMES  
ON THE *IN VITRO* DIGESTIBILITY OF SOYBEAN PROTEIN

| Sample                | Experiment |                                                                                      | Extent of hydrolysis |              |
|-----------------------|------------|--------------------------------------------------------------------------------------|----------------------|--------------|
|                       | No         | Description                                                                          | After 1 day          | After 5 days |
| Raw soy flour         | 1          | Incubated with pancreatin                                                            | %<br>10              | %<br>18      |
|                       | 2          | Incubated without pancreatin                                                         | 1                    | 3            |
|                       | 3          | Heated in aqueous suspension for 30 minutes at 60°C., then incubated with pancreatin |                      |              |
|                       | 4          | Same as 3, but without pancreatin                                                    | 6<br>0               | 13<br>1      |
|                       | 5          | Heated in aqueous suspension for 30 minutes at 80°C., then incubated with pancreatin |                      |              |
|                       | 6          | Same as 5, but without pancreatin                                                    | 8<br>0               | 16<br>0      |
|                       | 7          | Heated in aqueous suspension for 30 minutes at 95°C., then incubated with pancreatin |                      |              |
|                       | 8          | Same as 7, but without pancreatin                                                    | 10<br>0              | 20<br>0      |
| Bakery-type soy flour | 9          | Incubated with pancreatin                                                            | 5                    | 10           |
|                       | 10         | Incubated without pancreatin                                                         | 0                    | 0            |
|                       | 11         | Same as 3                                                                            | 6                    | 10           |
|                       | 12         | Same as 3, but without pancreatin                                                    | 0                    | 0            |
|                       | 13         | Same as 5                                                                            | 8                    | 16           |
|                       | 14         | Same as 5, but without pancreatin                                                    | 0                    | 0            |
|                       | 15         | Same as 7                                                                            | 10                   | 20           |
|                       | 16         | Same as 7, but without pancreatin                                                    | 0                    | 0            |

proteases in the test system, but this effect, contributing to decreased digestibility, was masked by the concomitant denaturation of the soy proteins rendering them more susceptible to *in vitro* pancreatic digestion.

It was apparent that, in order for the *in vitro* digestibility test to be applied to the characterization of soy products with respect to the nutritional value of the protein component, it would be necessary to



render inactive any proteolytic enzymes that might be naturally present. To accomplish this, the soy flour samples were subjected to a standardized heat treatment in the laboratory prior to being assayed for *in vitro* digestibility. Samples containing 6 g. of protein were suspended in 60 ml. of water and the suspensions heated in a boiling water bath for 15, 30, and 60 minutes. Susceptibility of the protein to *in vitro* enzymic digestion was determined in the previously described manner. The higher temperature was selected to assure

TABLE IV

INFLUENCE OF STANDARDIZED LABORATORY HEAT TREATMENT\* ON THE *IN VITRO* DIGESTIBILITY OF COMMERCIALY AVAILABLE SOYBEAN FLOURS IN VARIOUS STAGES OF HEAT-PROCESSING

| Sample No                           | Standardized laboratory heat treatment | Extent of hydrolysis |              |              |
|-------------------------------------|----------------------------------------|----------------------|--------------|--------------|
|                                     |                                        | After 1 day          | After 2 days | After 5 days |
| 1<br>Raw flour                      | <i>minutes</i>                         | %                    | %            | %            |
|                                     | None                                   | 12                   | 16           | 22           |
|                                     | 30                                     | 12                   | 15           | 23           |
| 2<br>Raw flour                      | None                                   | 12                   | 15           | 20           |
|                                     | 15                                     | 12                   | 15           | 20           |
|                                     | 30                                     | 13                   | 15           | 20           |
|                                     | 60                                     | 13                   | 16           | 21           |
| 3<br>Bakery-type flour              | None                                   | 6                    | 9            | 15           |
|                                     | 15                                     | 10                   | 13           | 20           |
|                                     | 30                                     | 13                   | 17           | 22           |
|                                     | 60                                     | 15                   | 18           | 23           |
| 5<br>Optimally heat-processed flour | None                                   | 13                   | 19           | 23           |
|                                     | 15                                     | 14                   | 18           | 24           |
|                                     | 30                                     | 15                   | 18           | 24           |
|                                     | 60                                     | 15                   | 20           | 24           |
| 5<br>Overheated flour               | None                                   | 16                   | 20           | 29           |
|                                     | 15                                     | 21                   | 25           | 29           |
|                                     | 30                                     | 22                   | 25           | 28           |
|                                     | 60                                     | 22                   | 26           | 29           |

\* Aqueous suspension of 1 part flour in 6 parts of water heated in a boiling water bath for the indicated length of time. The temperature of the suspension was approximately 95°C.

complete inactivation of the naturally-occurring enzymes, since the retention of only a small amount could result in relatively large increases in *in vitro* digestibility due to synergism with pancreatin.

Shown in Table IV are the results of these experiments. Although the *in vitro* digestibility of the raw product did not appear to be significantly improved by this laboratory heat treatment, it should be recognized that the digestibility prior to the heat treatment was in part due to the synergism between the naturally-occurring proteolytic enzymes and the added pancreatin. The change in *in vitro* digestibility

due to the standardized laboratory heat treatment should more properly be measured from the otherwise lower value that would have been obtained in the absence of naturally-occurring proteolytic activity as indicated in Table III. Taking this into account, the increase in *in vitro* digestibility, though not apparent here, would indeed be large.

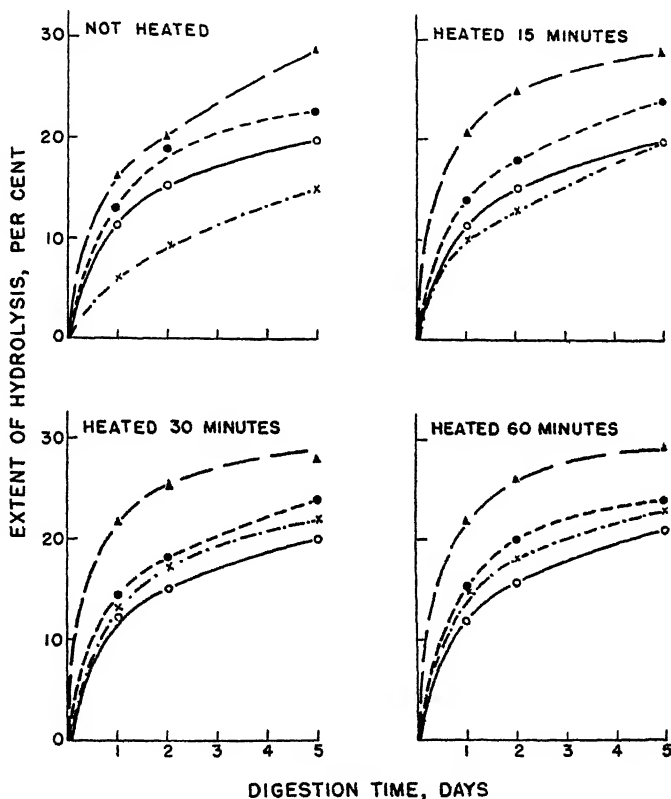


Fig. 1. Influence of standardized laboratory heat treatment on the *in vitro* digestibility of commercially heat-processed soybean products.

- O—Raw soy flour (Sample No. 2)
- X—Mildly heated bakery-type flour (Sample No. 3)
- Heated to optimal protein efficiency (Sample No. 4)
- ▲—Overheated with respect to protein efficiency (Sample No. 5)

The bakery-type product, whose naturally-occurring proteolytic activity had been inactivated by commercial heat processing, exhibited considerable improvement as a result of the laboratory heat treatment. After having been heated for 30 minutes, its digestibility value even exceeded, although to a small degree, that of the similarly heated raw product. In the case of the optimally heat-processed product only a slight improvement was observed; while in the over-

heated sample the improvement occurred in the first 15 minutes of the laboratory heat treatment, with no further change upon extending the heating time to 60 minutes. It will be noted in Fig. 1 that when the soy flour suspensions in water were subjected to the standardized laboratory heat treatment (95°C.) for periods of 30 and 60 minutes prior to being assayed, the *in vitro* digestibility values obtained were in the same sequence as the degree of commercial heat-processing. The differences in *in vitro* digestibility thus obtained, however, are not sufficiently great to permit rating soy products with respect to the nutritive value of the protein.

It will be observed that when no standardized laboratory heat treatment was employed, *in vitro* digestibility differences among samples 3, 4, and 5 were relatively large. Sample 2, the raw product, was anomalously high due to the presence of the naturally-occurring proteolytic enzymes which exerted a synergistic effect in the *in vitro* test. It is conceivable that with the application of very mild heat (60°C. or less) for suitable periods of time, the naturally-occurring proteolytic enzymes could be rendered inactive without effecting any alterations in the characteristics of the soybean protein *per se*, which affect digestibility. Other procedures such as using dry heat at higher temperatures might also prove of value in achieving this effect. With the interfering factor thus removed, *in vitro* proteolysis would be due to added pancreatin alone. No change would be anticipated in the *in vitro* digestibility of the proteins of those products which had been sufficiently heat-processed commercially to render inactive the proteolytic enzymes, since the mild laboratory heat treatment would be insufficient to cause further protein denaturation. In the case of the raw product, however, the *in vitro* digestibility value would be expected to be lower than that of sample 3 (the bakery-type product), due to the inactivation of the soybean proteases.

It has been demonstrated in the present study that a soy product (the bakery-type) can be heat-processed in such manner as to improve appreciably the nutritional value of the protein without impairment of its anti-tryptic activity *in vitro*. In this respect, the product resembles spray-dried egg albumen, which exhibits both high nutritional value and high anti-tryptic activity *in vitro* (1). Borchers *et al.* (6) have demonstrated that with increasing laboratory manipulation, it was possible to obtain a highly concentrated soybean trypsin inhibitor (by *in vitro* measurement), with insignificant growth-depressing properties. It must not be overlooked that each of the steps involved in the extended laboratory process permits the occurrence of some protein denaturation. Under such conditions of incipient protein denaturation, it is possible that certain properties of the anti-

tryptic complex (viz., those responsible for growth inhibition) may be altered while others remain unchanged (viz., anti-trypsin activity *in vitro*). Another likely explanation may be that the inhibitor complex, in its condition of incipient denaturation, becomes susceptible to digestion by pepsin so that its anti-tryptic activity *in vivo* is no longer apparent. It has been demonstrated by Kunitz (23) that the soy inhibitor in its native state is scarcely affected by pepsin at pH 3.0. When denatured, however, it is readily digested. At a pH of 2.0 or lower, the native inhibitor becomes slowly digestible at a rate less than 1/500 of that of the denatured material. This digestibility of the native inhibitor by pepsin has been attributed to the slight denaturation effected by the acid conditions.

The possibility of coupling the results of the *in vitro* digestibility studies with those obtained in our laboratories in evaluating the soy-curd potentialities of soy products (25) also merits consideration. The ideal soy product, with respect to protein value, should be readily susceptible to *in vitro* digestion in a test system free from naturally-occurring soy proteases and should yield a soy curd low in volume and capable of being further reduced when the sample is subjected to further heat treatment. These approaches to the over-all problem of characterizing soy products for specific functions are now under investigation at the Quartermaster Food and Container Institute for the Armed Forces.

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# LABORATORY PROCEDURE FOR EVALUATING THE CURD-PRODUCING CAPACITY OF SOYA PRODUCTS<sup>1</sup>

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## ABSTRACT

A procedure for the rating of soya products as sources of soy curd, employing the essential steps of Asiatic soy curd processing, involves the aqueous extraction of the soy protein and its precipitation as a curd on the addition of magnesium chloride, the curd being measured volumetrically.

Appreciably lower curd yields are obtained from material larger than that which passes through a No. 100 U. S. Standard wire sieve. Maximal solubility and precipitation of protein is obtained when the extraction is conducted at a temperature of 80°C. for a period of thirty minutes with the suspension stirred mechanically. Salts of strong acids such as calcium chloride, magnesium chloride, ferric chloride, and sodium bisulphate are effective agents for the precipitation of soy proteins. Hydrochloric acid is also an effective curdling reagent. Maximal yields of soy curd using magnesium chloride as the precipitating reagent occur at pH 5.8, whereas isoelectric precipitation using hydrochloric acid is at pH 4.5. Excessive quantities of the salts used to precipitate the soy proteins have given, within the concentrations studied, smaller yields of soy curd, the decreased yields being a characteristic of the precipitating salt not necessarily correlated with the pH of the final medium.

Data are presented showing that curd volume is an accurate index to the percentage of soluble protein in the soy flours.

The use of soya in relief feeding in both Europe and the Orient created new problems in description and control of products manufactured for different purposes. In Europe soya is used as a wheat flour extender, as a meat extender, and as a major ingredient for low cost food preparations; in the Orient it is used for the manufacture of soy sauce derived from the hydrolysis of protein, for "tofu" or soy curd prepared by the precipitation of water-extractable protein, and for "miso" or soy paste.

The present specification for soya products, Joint Army-Navy Specification JAN-S-588, refers to the protein as simply  $N \times 6.25$ , no consideration being given to the functional or nutritional properties of the foodstuff. A product may be satisfactory for one purpose, but

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fail in another. The evaluation of soya products utilized in the preparation of curd presented one of the greatest problems. This study concerns the development of a precise and rapid laboratory procedure for this purpose.

### Materials and Methods

Samples of soy flour, representative of raw and heat-treated commercial products, were kindly furnished by members of the Soy Flour Association. In addition, Dr. James W. Hayward of Archer-Daniels-Midland Company, Minneapolis, Minnesota, in behalf of the Soy Flour Association, supplied data obtained in their analyses of the products according to conventional procedures.

The products consisted of (1) dehulled soy beans, (2) unheated solvent-extracted soy flour, (3) bakery type soy flour, mildly heat-processed in order to eliminate the bitter qualities of the raw bean, (4) soy flour optimally heat-processed with respect to the nutritive quality of the protein, and (5) an overheated processed flour experimentally processed for this study. These raw materials for flours were supplied in flake form. In addition, an expeller series was supplied, consisting of (6) dehulled beans which had been heat-treated, (7) expeller flour with some additional heating, (8) flour, heat-processed so the protein had optimum biological value, and (9) overheated soy flour. These samples were supplied in chip form.

Samples were found to be most easily ground with a W. J. Fitzpatrick Comminuting mill. Solvent extracted flakes were ground through a 1 mm. mesh sieve and then reground through a No. 40 wire sieve. Samples 1, 6, 7, 8, and 9, because of their appreciable fat content, were ground through a 1 mm. mesh sieve and then extracted with Skelly Solve "B" in a Soxhlet extractor for 16 hours. After thorough air-drying these were reground through a No. 40 wire sieve.

Optimum sifting time for all samples through a No. 100 U. S. Standard sieve was determined by the method of Wichser, Shellenberger, and Pence (7). All samples were sifted through a No. 100 wire sieve.

In the many soy curd methods used by the Orientals, the essential steps are (1) aqueous extraction of soluble protein, (2) separation of solution from residue, and (3) precipitation of protein by means of salts, mainly those of magnesium. The manner in which these steps are performed are many and varied but the results are comparable. When the whole bean is employed a soaking step may be utilized to remove the hulls and facilitate wet grinding. Time and temperature for extraction of the crushed or milled bean may vary widely from a few

minutes in boiling water to several hours in hot water. Separation of solution from residue is accomplished by decantation or filtration through a cloth. Precipitation methods employed by the Orientals differ as to the reagent used; however, salts of calcium and magnesium, i.e. gypsum, magnesium sulphate, calcium chloride, magnesium chloride, and mixtures derived from evaporation of sea water, are used almost exclusively. To establish reproducible conditions for the above steps the following basic procedure was used:

The extract was prepared by suspending 8.00 g. of soy flour in 100 ml. of distilled water, or multiple of this flour-water ratio, and stirred with a mechanical stirring device. After extraction the suspension was centrifuged in an International centrifuge No. 1 with trunnion cups, radius measurement 20 cm. to the tip, 1,800 r.p.m. for ten minutes. Twenty-five ml. of the extract liquid were pipetted into a 50 ml. long-tapered centrifuge tube graduated in fractions of ml. to 20 ml.; curdling solution was added and the suspension diluted to the 50 ml. mark. Centrifuging was again accomplished at 1,800 r.p.m. for ten minutes.

### Results and Discussion

*Influence of Temperature on the Extraction of the Soy Proteins.* Sample No. 3, bakery type soy flour, was selected for this study. Of the samples submitted, it contained an intermediate quantity of

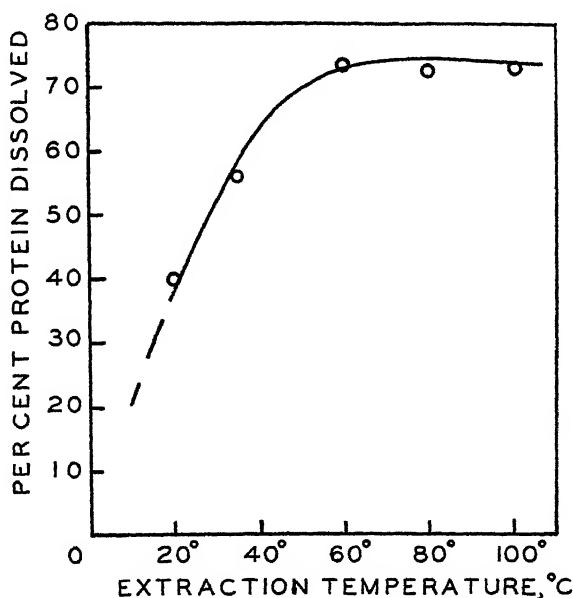


Fig. 1. The relationship of soluble protein and temperature of extraction; time, 60 min.; test material, bakery type soy flour (Sample No. 3).



soluble protein. The flour was extracted at several temperature levels for one hour. Protein analyses ( $N \times 6.25$ ) of 25 ml. aliquots of the extract, after centrifugation, indicate maximum solubility of the protein at 60°C. to 100°C. as shown in Fig. 1. In another experiment designed to determine the effect of time, extractions were repeated at 80°C. for intervals of 10, 20, 30, 40, and 60 minutes. Fig. 2 shows that at 20 minutes, extraction has reached a maximum with no significant change on continued heating. Since 30 minutes and 80°C. extraction conditions are sufficient to insure maximum protein dispersion, a longer extraction period is unnecessary and undesirable because of

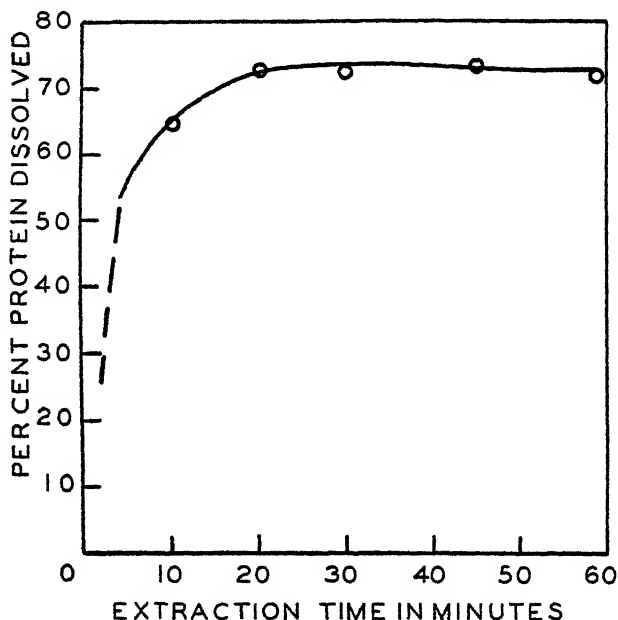


Fig. 2. The relationship of soluble protein, extracted at 80°C., to time, test material, bakery type soy flour (Sample No. 3).

water evaporation; this can lead to an erroneously high estimate of soluble protein.

Although the yield of soluble protein by this method of hot extraction was greater than that reported by the manufacturer for this sample,<sup>4</sup> viz. 70% as compared to 65%, it did not exceed the manufacturer's reported value for the raw sample (No. 2). This increased yield of soluble protein was evident in all samples and particularly in the heat treated soya products.

*Optimum Concentrations of Curding or Precipitating Reagents.* Several salts and hydrochloric acid were evaluated as precipitating

<sup>4</sup> Indirect Method; Archer-Daniels-Midland Company

agents in this study. Sample No. 2, unheated soy flour, which exhibited greatest solubility, was used for the tests. Protein from 25 ml. of extract was precipitated with solutions of magnesium chloride, ferric chloride, sodium bisulphate, and hydrochloric acid. The final normality after dilution to 50 ml. was calculated. By subtracting twice the protein content of 25 ml. of supernatant solution after precipitation and centrifugation of the curd from the protein content of the extract, the weight of protein in the curd can be determined.

Fig. 3 shows that maximal yield of precipitated protein was attained when the solutions were 0.01 *N* to 0.03 *N* with respect to the salt and

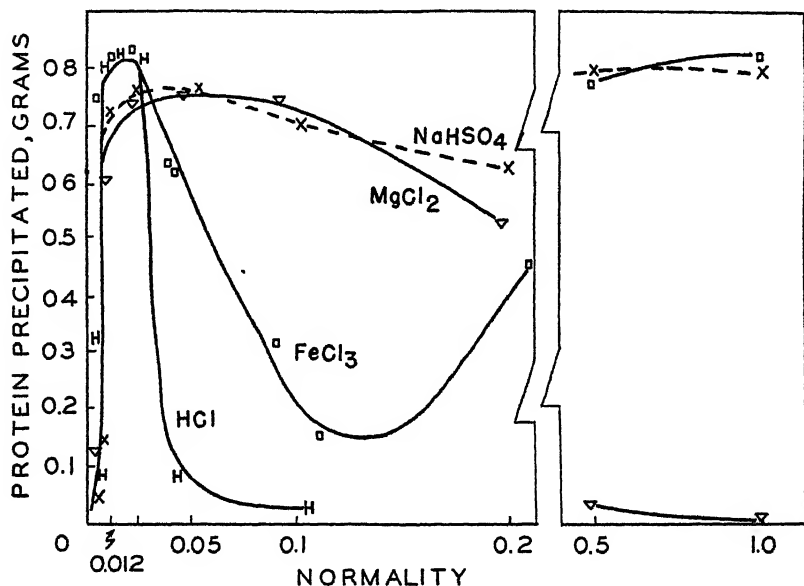


Fig. 3. Relation of salt and acid concentration to the yield of precipitable protein of soya; test material, defatted raw soya (Sample No. 2).

acid. This is in close agreement with the results of Smith *et al.* (6) who report minimum extraction of soybean nitrogen with calcium chloride solution of 0.0175 *N* and magnesium chloride solution of 0.025 *N*. Minimum dispersion of nitrogenous matter in acid was obtained at pH 4.2 by these authors (5). Maximum protein precipitated by hydrochloric acid was found in this study to be in the pH range of 5.0 to 4.0. Hydrochloric acid or isoelectric precipitation was noted to be slightly more efficient than magnesium chloride, yielding approximately 4% more of the available protein. The curves plotted in Fig. 4 indicate that the yield of curd is not necessarily correlated with the pH of the final medium.

*The Effect of Heating upon the Solubility of the Protein and upon the Precipitation of the Curd.* The data in Table I illustrate the effect of cold extraction and of hot extraction followed by curding from cold and hot solutions. Extraction at room temperature is not efficient under the conditions described. The curd volume was less than anticipated compared to the amount of protein in solution. The condition of the curd was fluid in nature and the residual protein after curding was large. By slightly heating the extract after the first centrifugation a more efficient precipitation was obtained, denoted by an increase in curd volume and decrease in quantity of protein in the top liquid.

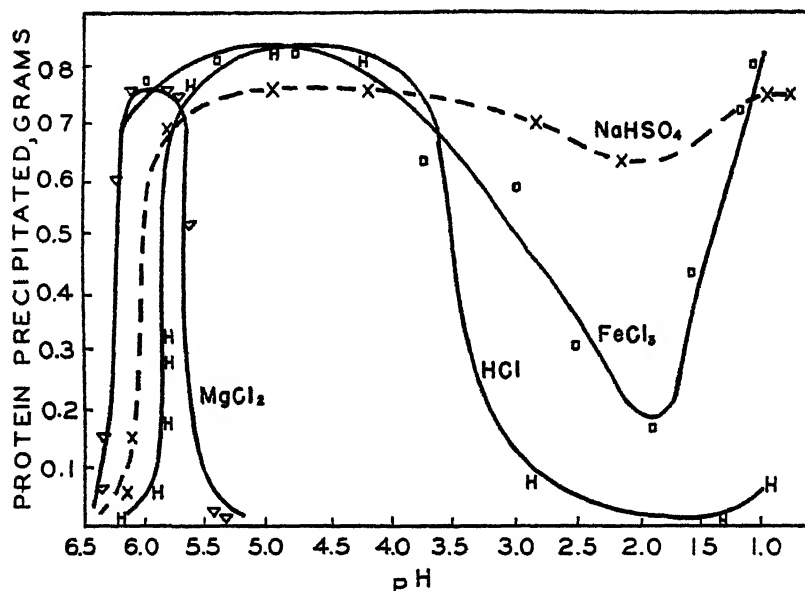


Fig 4. The relationship of the yield of curd to the pH of three salt solutions and hydrochloric acid; test material, delatted raw soya (Sample No. 2).

Thirty minutes extraction at 80°C. increases the yield of soluble protein (as was shown in Fig. 1), but cooling the extract at room temperature before protein precipitation gives a curd having undesirable small creamy particles which do not pack well, resulting in an apparently greater volume. Heating serves a two-fold purpose: (1) it produces the maximum yield of soluble protein in the minimum of time, and (2) causes incipient denaturation of the extractable protein, making it more readily precipitable following the addition of the curding reagent. The heat treatment yields a curd possessing good consistency and which is easily centrifuged, thereby improving the precision of the gravimetric separation.

*The Effect of Granulation or Particle Size of Flour on Extraction of Protein and on Soy Curd Yield.* Marked changes in curd yield by volumetric measurement were noticed with the same soy flour of different granulation. Samples of the unheated (Sample No. 2) and the optimally-heated (Sample No. 4) soy flours were used to determine the effect of granulation or particle size on the reproducibility of the

TABLE I  
EFFECT OF HEATING UPON THE SOLUBILITY OF THE PROTEIN AND  
UPON THE PRECIPITATION OF THE CURD<sup>1</sup>

| Extraction and curding method                                                | Curding agent     | pH curd suspension | Curd vol. | Distribution of protein <sup>2</sup> |      |              |
|------------------------------------------------------------------------------|-------------------|--------------------|-----------|--------------------------------------|------|--------------|
|                                                                              |                   |                    |           | Extract                              | Curd | Super-natant |
| Two hrs. extract at 23°C.<br>Curding at 23°C.                                | MgCl <sub>2</sub> | 5.65               | 1.8       | 61.6                                 | 41.3 | 20.3         |
|                                                                              |                   | 5.65               | 1.9       |                                      | 40.1 | 21.6         |
|                                                                              | HCl               | 4.80               | 1.9       |                                      | 47.6 | 14.0         |
|                                                                              |                   | 4.80               | 1.9       |                                      | 47.9 | 13.8         |
| Two hrs. extract at 23°C.<br>Brought to 80°C. before curding                 | MgCl <sub>2</sub> | 5.70               | 3.5       | 64.2                                 | 53.3 | 10.9         |
|                                                                              |                   | 5.70               | 4.0       |                                      | 53.3 | 10.9         |
|                                                                              | HCl               | 4.80               | 3.5       |                                      | 56.3 | 7.9          |
|                                                                              |                   | 4.80               | 3.5       |                                      | 56.1 | 8.1          |
| Two hrs. extract at 23°C.<br>Protein solution heated 30 min. at 80°C.        | MgCl <sub>2</sub> | 5.68               | 5.5       | 64.0                                 | 53.8 | 10.2         |
|                                                                              |                   | 5.65               | 5.5       |                                      | 53.8 | 10.2         |
|                                                                              | HCl               | 4.60               | 5.5       |                                      | 56.7 | 7.3          |
|                                                                              |                   | 4.60               | 5.5       |                                      | 56.7 | 7.3          |
| Thirty min. extract at 80°C.<br>Protein solution cooled to 23°C. for curding | MgCl <sub>2</sub> | 5.70               | 7.5       | 86.6                                 | 74.3 | 12.3         |
|                                                                              |                   | 5.70               | 7.5       |                                      | 74.3 | 12.3         |
|                                                                              | HCl               | 4.60               | 7.0       |                                      | 78.2 | 8.4          |
|                                                                              |                   | 4.60               | 7.0       |                                      | 78.7 | 7.9          |

<sup>1</sup> Defatted raw soy flour (Sample No. 2) used in this study, 8 g. flour to 100 ml. water.

<sup>2</sup> Per cent protein calculated as per cent of total protein. Supernatant protein determined by Kjeldahl method on an aliquot of top liquid after precipitation. Protein of curd calculated by difference; extract protein minus protein of supernatant liquid after precipitation.

test. The Joint Army-Navy Specification for Soybean Products, dated May 10, 1948 specifies:

"E-7. Ninety-seven per cent of the soy flour shall pass through a U. S. Standard 100-mesh screen."

The materials through and over a 100-mesh screen of Samples No. 2 and No. 4 were tested for soluble protein and curd yield. The results in Table II, average of duplicate tests, emphasize that large differences in per cent soluble protein and in curd volume are obtainable if the

particle size of the flour is not controlled. The per cent of protein of the initial soy flour fractions was nearly the same. Therefore, the difference in curd yield is attributed to the greater solubility of the material passing through the 100-mesh screen, due to larger surface area and mechanical damage to cell structure. To insure maximum solubility of protein at the time and temperature of the test all samples should be ground finer than 100-mesh.

TABLE II  
EFFECT OF GRANULATION OR PARTICLE SIZE OF  
FLOUR ON SOY CURD YIELD

| Sample             | Curd vol. | Distribution of protein <sup>1</sup> |              |             |
|--------------------|-----------|--------------------------------------|--------------|-------------|
|                    |           | Extract                              | Curd (calc.) | Supernatant |
|                    | ml.       | %                                    | %            | %           |
| Unheated soy       | 5.00      | 73.40                                | 57.95        | 15.45       |
| Sample No. 2       | 5.00      | 74.02                                | 58.98        | 15.03       |
| Over 100w          | 5.00      | 72.46                                | 57.64        | 14.82       |
|                    | 5.00      | 74.22                                | 59.19        | 15.03       |
| Mean               | —         | 73.52                                | 58.44        | 15.08       |
| Standard deviation | —         | ± 0.71                               | —            | ± 0.29      |
| Unheated soy       | 6.25      | 84.90                                | 73.08        | 11.82       |
| Sample No. 2       | 6.25      | 83.85                                | 72.02        | 11.82       |
| Through 100w       | 6.25      | 85.19                                | 73.55        | 11.64       |
|                    | 6.25      | 84.71                                | 72.79        | 11.92       |
| Mean               | —         | 84.66                                | 72.86        | 11.80       |
| Standard deviation | —         | ± 0.55                               | —            | ± 0.12      |
| Heated soy         | 1.80      | 27.30                                | 17.70        | 9.60        |
| Sample No. 4       | 1.80      | 27.50                                | 18.00        | 9.50        |
| Over 100w          | 1.80      | 27.30                                | 17.70        | 9.60        |
|                    | 1.80      | 27.60                                | 18.30        | 9.30        |
| Mean               | —         | 27.42                                | 17.92        | 9.50        |
| Standard deviation | —         | ± 0.14                               | —            | ± 0.14      |
| Heated soy         | 2.30      | 36.24                                | 26.95        | 9.29        |
| Sample No. 4       | 2.30      | 36.06                                | 26.77        | 9.29        |
| Through 100w       | 2.30      | 35.31                                | 26.30        | 9.01        |
|                    | 2.30      | 35.40                                | 26.21        | 9.19        |
| Mean               | —         | 35.75                                | 26.56        | 9.19        |
| Standard deviation | —         | ± 0.47                               | —            | ± 0.13      |

<sup>1</sup> Per cent protein calculated as per cent of total protein.

*Correlation of Curd Volume with Amount of Protein Precipitated.* Initial determinations on the nine samples included in this study indicated a variation in soluble protein content; three samples possessed a high proportion of soluble protein while the other six had smaller amounts. To determine if curd volume, by the proposed method of magnesium chloride precipitation, is linearly related to protein precipitated, increasing quantities of extract of Sample No. 2 were diluted to 25 ml. and subjected to the curd test. The weight of

protein used in each case was a fraction of the amount of protein in the 25 ml. of extract. The data plotted in Fig. 5 show that this relation is linear with a correlation coefficient of  $r = +0.996^{**}$ .

The tests were extended to 11 samples and quantities of less than 25 ml. were used whenever the smaller aliquots would yield measurable curd volumes. For this reason aliquots less than 20 ml. were not practical in the cases of the heat-treated samples. Fig. 6 illustrates the relationship found with 35 aliquots of 11 soy samples; a correlation coefficient of  $r = +0.984^{**}$  and a regression equation

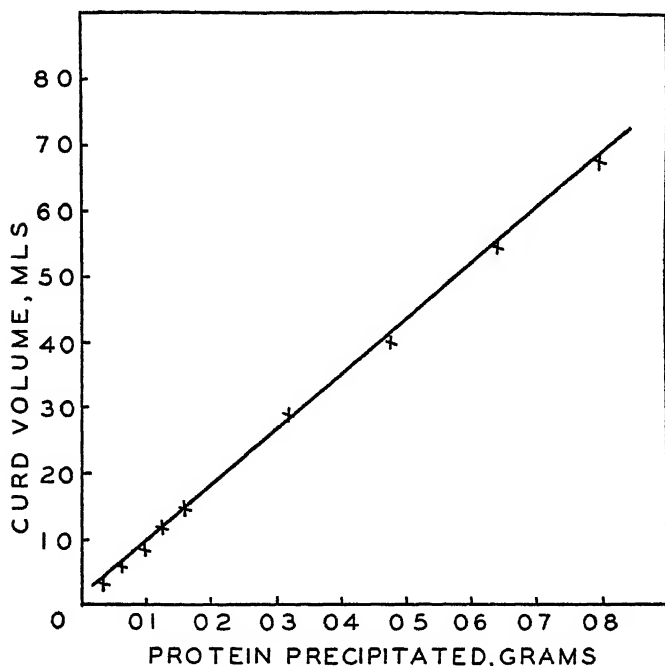


Fig 5. Relation of protein precipitated to curd volumes, test material, defatted raw soya (Sample No 2)

of  $Y = 7.26 X + 0.26$  were found, where  $Y$  = observed curd volume and  $X$  = grams of protein precipitated.

Included in these tests were three samples of soy meal. These were unsolicited samples from industry without accompanying analytical data and are identified as A, B, and C. Comparison with test samples indicates that A has been slightly heat-treated and was satisfactory for curd preparation. Heat treatment of samples B and C materially affected the solubility of the protein so that these would have to be used in formulations where functional properties of the protein are not important.

Plotting curd volume against per cent soluble protein and per cent of protein precipitated, Fig. 7, illustrates the relationship of soluble protein to the yield of curd. Soy flours of low protein solubility have a residual protein after precipitation of from 8 to 10%. High protein solubility flours have a residual protein of from 10 to 14%, indicated by the dotted lines.

The data from Table II were plotted on the figure, represented by the letters Z and Y. Letter Z represents material through a 100-mesh

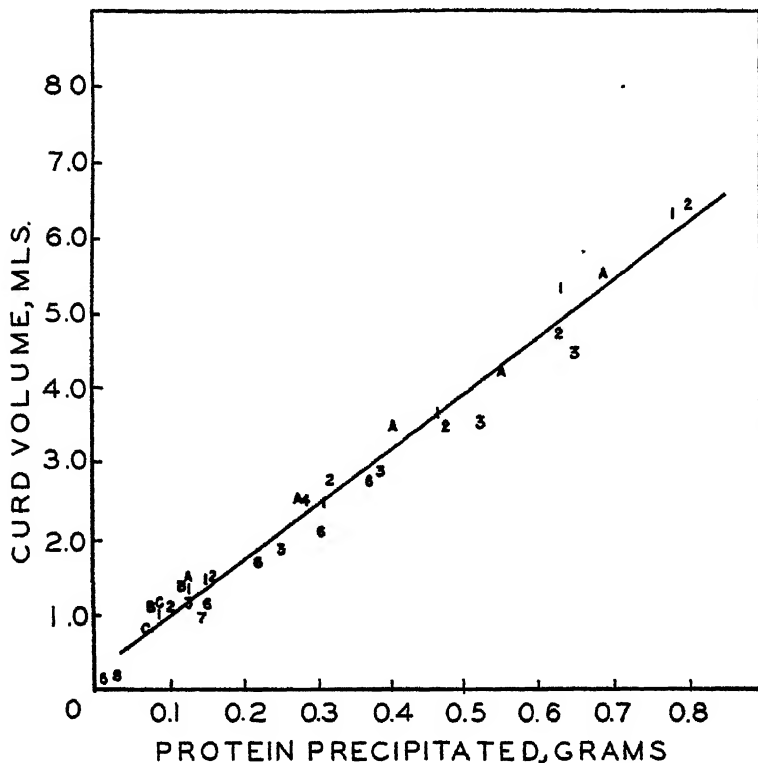


Fig. 6. Relation of protein precipitated to curd volume with thirty-five aliquots of eleven samples of soy flour and soy meal. The numbers refer to the soy flour test samples; the letters refer to solvent extracted soy meals.

sieve and Y the material over the wire from Samples No. 2 and No. 4. It is again evident that the yield of curd is dependent upon the amount of extractable protein.

A curd volume of not less than 4.0 ml. by this method for solvent-extracted soy flours and meals would identify such products as the type which would find acceptance with Orientals. Alternately, heat-treated products similar to samples 4 and 5 would be useful as far as

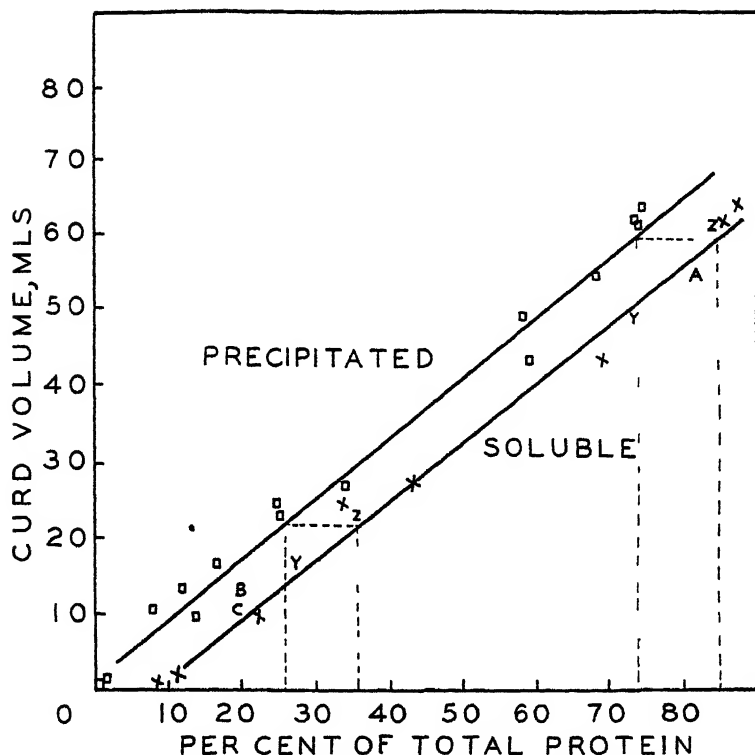


Fig 7 Relation of per cent soluble protein in soy products and per cent of protein precipitated to curd volume

consumption *in toto* is concerned, in soup mixes, and as protein supplements in cereals, but would be unsatisfactory for curd formation.

These studies have established that a good correlation exists between soy curd volume, as determined by this method, and quantity of protein precipitated, justifying the use of the far simpler volumetric procedure rather than the nitrogen analysis for the determination of the relative concentrations of the precipitable soy protein.

The method recommended is as follows:

#### Sample preparation:

Sample should be of such granulation that it passes through a No. 100-mesh U. S. Standard screen.

#### Apparatus:

- (1) Centrifuge bottles, Pyrex, 250 ml.
- (2) Pipettes, 25 ml.
- (3) Waterbath.
- (4) Mechanical or electrical stirring device with glass stirring rod.



- (5) Centrifuge, International No. 1 or equivalent, radius 8 in. (20 cm.) to tip, trunnion mount cups.
- (6) Centrifuge tubes, long taper, 50 ml., graduated in fractions of ml. to 20 ml.
- (7) Magnesium chloride solution, 0.20 *N* to 0.25 *N*.

#### Determination:

Weigh 8.00 g. soy product (8.0% moisture basis) and place in centrifuge bottle, add 100 ml. of distilled water and stir with glass rod from stirring device until all the material is in suspension and none adheres to the sides.

Place in water bath, 80°C.  $\pm$  2°C., and start stirring device. Extraction is continued 30 min. after temperature of the solution

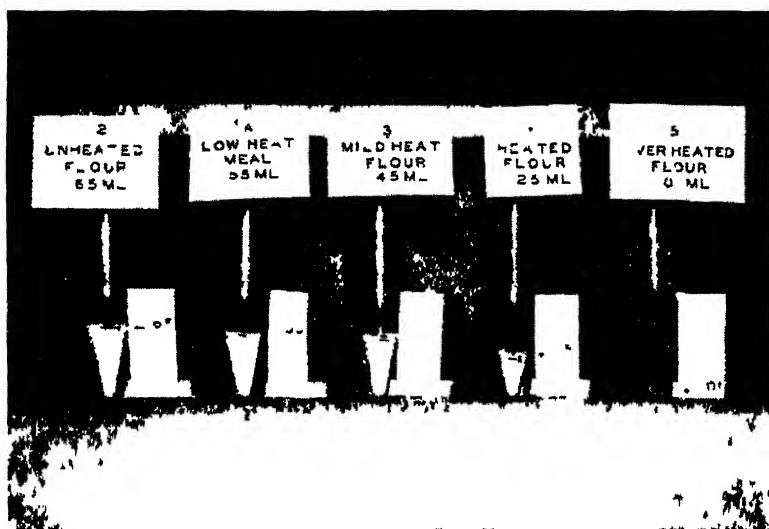


Fig 8 Illustration of five samples of defatted soya at the completion of the curd test

passes 60°C. (approximately 5 min.). Remove bottle and centrifuge at 1,800 r.p.m. for 10 min.

Pipette 25 ml. of the supernatant liquid into a long-tapered centrifuge tube, bring solution to 80°C., remove tubes and add 5 ml. of 0.2 *N* magnesium chloride solution while agitating the tube. Dilute to the 50 ml. mark with water and centrifuge at 1,800 r.p.m. for 10 min. Allow centrifuge to come to rest without braking and read curd volume to the nearest fraction of a ml. Two to three tests may be conducted on the extract for increased precision.

Fig. 8 illustrates the appearance of the centrifuge tubes at the completion of the curd test on five soybean products. The reproduci-

bility of the soy curd test, the ease of running such assays, and its direct applicability to evaluation of soya products intended for use in the Orient, justify inclusion of the method in an amendment to the present Joint Army-Navy Specification for the identification of soya products. In current investigations conducted in the Institute by Simon and Melnick (4), it has been suggested that the soy curd test has further value in defining heat-processed soya products with respect to the nutritive value of the protein.

It was previously mentioned that magnesium chloride precipitation of the soy protein occurs at a pH somewhat removed from the isoelectric point. Precipitation with hydrochloric acid yields a soy curd comparable to that formed following the use of magnesium chloride as the curding reagent but differs slightly in the yield of precipitated protein. In order to determine if the difference in curd yield was due to some protein fraction which was precipitated by hydrochloric acid at the isoelectric point and not precipitated by magnesium chloride, electrophoretic analysis was employed. A water extract of Sample No. 2 unheated soy flour was prepared according to the test method and divided into two portions. Magnesium chloride reagent was added to one portion and hydrochloric acid to the other in concentrations which produced maximum yield of curd. Electrophoretic analyses of the protein in the two supernatant solutions, kindly conducted by Dr. A. C. Shuman, General Foods Corporation, Hoboken, New Jersey, indicated that the two solutions were identical as far as their electrophoretic mobilities were concerned and each consisted of a single component with a small degree of non-homogeneity. Thus, precipitation at the isoelectric point yields the same protein precipitate as the procedures customarily employed by the Orientals.

In this connection, it is of value to speculate on the possible fate of the antitryptic factor in the raw or mildly-heated soya products which are of the type most favorable for soy curd formation. Such mildly-heated products contain the antitryptic factor, which has been held to be responsible for poor protein-utilization *in vivo* (2, 3). Precipitation with hydrochloric acid at the isoelectric point has been employed (1, 3) to remove irrelevant protein leaving in solution the antitryptic complex. It seems quite likely from the electrophoretic studies that the magnesium chloride reagent would also be effective for this purpose. However, the efficiency with which the antitryptic complex can be isolated by these procedures has not been adequately studied. The possibility of co-precipitation of the antitryptic complex with the soy curd does exist. This point of importance in predicting the value of the soy curd as a source of protein of high biological value is now under investigation in our laboratories.

### Acknowledgments

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## DETERMINATION OF IRON IN CEREALS, FLOUR, AND BREAD<sup>1</sup>

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### ABSTRACT

To determine iron in flour and bread, muffle ignition of a sample moistened with magnesium nitrate was found to be preferable to other ashing procedures because it prevents the loss of iron and offers simplicity and ease of manipulation. Results are reproducible to within 0.5%.

Possible interference by pyrophosphate is overcome by the presence of considerable quantities of magnesium, and by acid hydrolysis. Intensity and stability of the thiocyanate color complex with iron is increased by extraction with normal butyl alcohol.

Analyses were carried out on flour, bread, corn meal, macaroni, corn grits, and other cereal products. The initial iron content of the sample was ascertained and in each case additional iron was added and the recovery measured. In all instances this was found to be within 2.5% of the theoretical recovery. In some instances, addition of phosphate was also carried out, and in no event did this additional phosphate interfere with complete iron recovery.

Since iron is an important constituent of foodstuffs and is included in some form in the enrichment of cereals, flour, and bread, an analytical method for its determination in relatively small quantities is essential. Both the Association of Official Agricultural Chemists and the American Association of Cereal Chemists have taken cognizance

<sup>1</sup> Manuscript received September 19, 1949.

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of the problem involved by the publication of thoroughly tested methods for this analysis (2, 12).

In the official A.A.C.C. method specific for iron in flour, semolina and related products, 20 g. of unenriched flour or 5 g. of enriched flour, whole wheat or bread (2) are ashed overnight in a platinum dish at 550–600°C., the ash is dissolved in HCl, the iron reduced by hydroquinone and the ferrous iron then determined photocolormetrically by  $\alpha$ ,  $\alpha'$ -dipyridyl.

In the A.O.A.C. method for iron in cereal foods (12) a 10 g. sample is ashed in a platinum, silica, or porcelain dish at about 550°C., the ash is dissolved in HCl, reduced with hydroxylamine and the iron measured photometrically with either orthophenanthroline or  $\alpha$ ,  $\alpha'$ -dipyridyl. In this method, except for self-rising flour or bread, an ashing aid such as magnesium nitrate solution or nitric acid is recommended to be added to the initial ash if it is not obtained free of carbon. A recent report by Munsey (13) has confirmed the applicability of the A.O.A.C. method to the determination of iron in enriched spaghetti, enriched degerminated corn meal, and enriched corn meal.

During the past five years or more a generally applicable method based on the formation of ferric thiocyanate has been under investigation in our laboratories and has been found to yield results strictly in accordance with the two official methods outlined above. This method embodies certain modifications which are primarily time saving in nature. It has been investigated on commercially available samples of unenriched and enriched flour, self-rising flour, phosphated flour, whole wheat and rye flours, white, rye and whole wheat breads, enriched hominy grits, flaked grits, corn meal, a malted wheat cereal, doughnuts, doughnut mix, and macaroni. Interference of both calcium and phosphorus are circumvented.

A review of the literature reveals that the thiocyanate reagent is very widely used in the determination of small amounts of iron. The vast information on its use is in some cases contradictory. This reagent is particularly well suited for use on samples prepared with a high acid concentration, and was chosen for this reason. According to Woods and Mellon (21) the following variables must be kept reasonably constant: (a) amount and kind of acid, (b) excess quantity of oxidizing agent, (c) time of standing, (d) presence of interfering ions, and (e) dielectric constant of solvent.

Hallinan (7) has shown that a high concentration of hydrochloric acid is exceptionally well suited for the development of the ferric thiocyanate complex. Extraction by an immiscible solvent is of importance when small quantities of iron are to be determined. Isoamyl (15), isobutyl (17), and normal butyl alcohols appear to be ideal

solvents for concentrating, intensifying and stabilizing the color. The ferric thiocyanate complex extracted with isobutyl alcohol has a maximum absorption at 485  $m\mu$ ; with normal butyl alcohol at 490  $m\mu$  and the latter extraction is free from disturbances which occur when isoamyl alcohol is used (3). Winsor (19) has shown that the dielectric constant is satisfactory for the dissociation of the solute.

### Materials and Methods

Apparatus included: (1) A Coleman Double Monochromator and a Coleman Universal Spectrophotometer. (2) Pyrex Glassware—All glassware was cleaned with nitric acid and thoroughly rinsed with distilled water. (3) Silica Dishes—100 ml. silica dishes were cleaned with nitric acid and rinsed with distilled water.

Reagents consisted of: (1) A.C.S. reagent grade nitric and hydrochloric acids, potassium thiocyanate, and magnesium nitrate. Normal butyl alcohol, reagent grade, b.p. 116–118° was used without further treatment. (2) Butanolic potassium thiocyanate solution—100 g. of reagent grade potassium thiocyanate were dissolved in 100 ml. of water, heated to 25–30°C., diluted to 1000 ml. with n-butyl alcohol, shaken vigorously for at least 15 minutes and allowed to clear. (3) Magnesium nitrate—55 g. reagent grade magnesium nitrate,  $Mg(NO_3)_2 \cdot 6H_2O$ , were dissolved in sufficient distilled water to make 100 ml. of solution. (4) A stock standard iron solution was made by weighing 1.000 g. of reagent iron wire into a clean, iron-free beaker, dissolved in 20% hydrochloric acid to which 2 ml. of concentrated nitric acid were added. The solution was then carefully evaporated to dryness (on steam bath) and dissolved in 20 ml. of 10% hydrochloric acid, quantitatively transferred to a 1000 ml. volumetric flask and diluted to volume. This stock solution contained 1 mg. iron per ml. and was stable for relatively long periods of time when kept in a pyrex bottle. (5) The working standard was prepared by diluting 10 ml. of the stock solution to exactly 100 ml. with distilled water. One ml. contained 0.1 mg. iron (Fe).

A sufficient amount of finely powdered sample to contain about 0.1 mg. of iron was weighed into a 100 ml. silica dish. Exactly 10 ml. of magnesium nitrate solution were added and the mixture stirred with a glass rod until a uniform paste was obtained. The paste was spread in the dish and the rod wiped with a small piece of ashless filter paper, the latter being added to the sample. The paste was covered with about 5 ml. of alcohol and the dish transferred to a muffle furnace set at 600–650°C. The door of the furnace was left slightly open. When the ash turned entirely white or grayish-white (45 to 60 minutes), the dish was removed from the furnace and cooled. The ash was

carefully moistened with a fine stream of water and 3 ml. of nitric acid added and then evaporated to dryness on the steam bath. The ash was redissolved in a few ml. of water, 10 ml. of hydrochloric acid added and again evaporated on the steam bath almost to dryness. The residue was dissolved in 15 ml. 1 *N* hydrochloric acid and heated on the steam bath for about 15 minutes then cooled, transferred to a 50 ml. volumetric flask and diluted to volume with 1 *N* hydro-

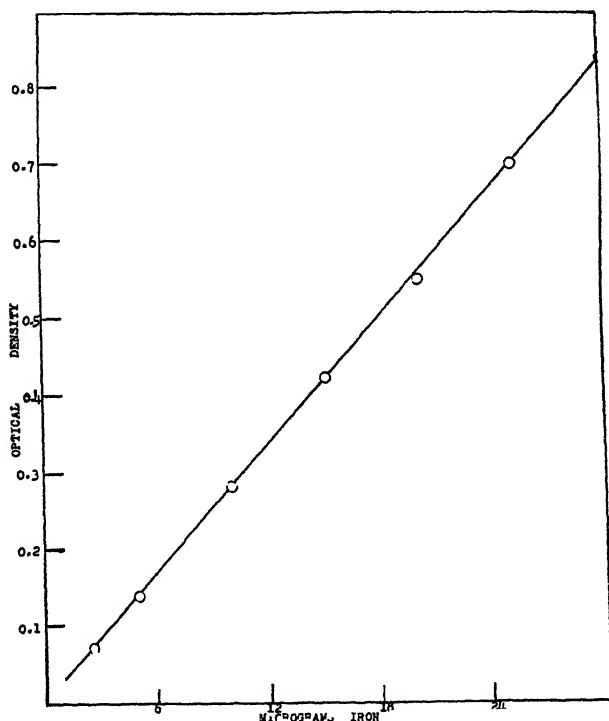


FIG. 1

chloric acid. If the resultant solution was not clear, it was filtered through a small filter, the first 15 ml. of filtrate being rejected.

A 10 ml. aliquot was transferred to a 60 ml. glass stoppered separatory funnel and exactly 15 ml. of butanolic potassium thiocyanate solution added, then shaken vigorously for 30 seconds and allowed to stand for five minutes to permit the two phases to separate. The aqueous layer was drawn off and discarded. The funnel was inverted and slowly revolved so as to dislodge any water particles that clung to the walls of the funnel and permitted to stand for five minutes. The small amount of water which separated from the alcohol was drawn

off and the alcohol layer transferred to a suitable cuvette. Readings were made at 490  $m\mu$ , with the blank set at 100% transmission. The separation of the two phases was carefully controlled. The ratio of alcohol to water after the first separation became very great, and at this point a more than gentle revolving of the funnel produced an emulsion. This resulted in a slow separation of water and caused erratic results. Centrifuging corrected this difficulty.

A 10 ml. aliquot was transferred to a 60 ml. glass-stoppered funnel, 15 ml. of *n*-butyl alcohol added, but no thiocyanate, shaken vigorously for 30 seconds and allowed to stand for five minutes to permit the two phases to separate. The aqueous layer was drawn off and discarded. The alcoholic layer was transferred to a suitable cuvette and set for 100% transmission.

A standard curve was prepared by transferring 0.25, 0.5, 0.75, 1.0, 1.25, and 1.5 ml. of the standard solution to silica dishes, adding exactly 10 ml. of magnesium nitrate solution and 3 ml. of nitric acid to each and evaporating to near dryness on the steam bath. These were treated exactly as the sample.

The standard curve was checked each time fresh solutions of thiocyanate and magnesium nitrate were prepared. This was done by verifying at least two points on the curve. A typical curve is shown in Fig. 1.

### Discussion

In an attempt to find the most suitable ashing procedure, the following were tried: dry ashing and fusion of the ash with sodium carbonate (1), dry ashing with small amounts of sulfuric acid (10), dry ashing with sodium hydroxide (8), dry ashing with calcium carbonate (6), digestion with sulfuric, nitric, and perchloric acids (9), and ashing after wetting with solution of magnesium nitrate. The first three procedures gave consistently low results. Acid digestion gave satisfactory results after a long and tedious operation. It was found that if the sample of flour, bread, or cereal was thoroughly mixed to form a

TABLE I  
IRON RECOVERED FROM ENRICHED \* PATENT FLOUR  
(Mg. per pound)

| Dry ashing and<br>$\text{Na}_2\text{CO}_3$ fusion | $\text{CaCO}_3$<br>ashing | $\text{H}_2\text{SO}_4$<br>ashing | $\text{NaOH}$<br>ashing | Wet<br>ashing | $\text{Mg}(\text{NO}_3)_2$<br>ashing |
|---------------------------------------------------|---------------------------|-----------------------------------|-------------------------|---------------|--------------------------------------|
| 9.27                                              | 13.05                     | 11.40                             | 12.60                   | 13.60         | 13.96                                |
| 11.44                                             | 12.85                     | 13.00                             | 12.05                   | 13.75         | 13.73                                |
| 10.48                                             | 13.28                     | 10.80                             | 13.02                   | 13.85         | 13.88                                |

\* Enriched to contain 13 mg. iron per pound.

TABLE II  
IRON RECOVERY IN PRESENCE OF PYROPHOSPHATE  
20  $\mu$ g. Fe and 50 mg.  $P_2O_5$  (added as  $Na_4P_2O_7$ )  
(Solutions made up in N/1 HCl)

| Magnesium present | Aluminum present | Fe recovered before hydrolysis | Fe recovered after hydrolysis |
|-------------------|------------------|--------------------------------|-------------------------------|
| $m_2$             | $mg$             | $\%$                           | $\%$                          |
| 0                 | 0                | 12.5                           | 94.5                          |
| 50                | —                | 50.5                           | 98.0                          |
| 100               | —                | 70.0                           | 98.6                          |
| 250               | —                | 93.0                           | 98.6                          |
| 350               | —                | 93.5                           | 98.5                          |
|                   | 10               | 86.5                           | 97.0                          |
|                   | 50               | 97.0                           | 98.6                          |
|                   | 100              | 98.5                           | 98.6                          |
|                   | 200              | 98.5                           | 98.5                          |

paste with magnesium nitrate solution and ignited, consistently good checks and iron recoveries were obtained. The addition of the magnesium nitrate prior to the initial ashing of the sample permits a more rapid ashing with little or no difficulties. The ash is always free of carbon and necessity of reigniting a sample is eliminated. Silica dishes were used and a grayish-white ash from 10 g. of flour was obtained in 45 minutes. As a final test a sample of an enriched flour was ashed in triplicate by each of the above-mentioned procedures. The iron content of the ash after acid hydrolysis was determined by extraction with potassium thiocyanate in n-butyl alcohol solution. The results are given in Table I.

After numerous experiments, it was found that the formation and extraction of the color complex could be accomplished in one operation

TABLE III

| Sample                         | A O A C.<br>mg. Fe/lb. found | KCNS<br>mg. Fe/lb | Proposed ignition<br>and $\alpha$ , $\alpha'$ -dipyridyl |
|--------------------------------|------------------------------|-------------------|----------------------------------------------------------|
| Enriched flour                 | 14.0                         | 14.0              | 14.3                                                     |
| Enriched self-rising flour (A) | 19.3<br>19.8                 | 19.7<br>19.4      | 19.5                                                     |
| Enriched self-rising flour (B) | 13.0<br>12.6                 | 12.7<br>12.8      | 13.2                                                     |
| Enriched corn meal             | 20.6<br>20.0                 | 20.3<br>20.0      | 20.6                                                     |
| Macaroni                       | 19.0<br>18.0                 | 18.7<br>18.9      | 18.4                                                     |
| Corn grits                     | 21.2<br>21.8                 | 21.1<br>21.4      | 21.2                                                     |



by shaking the acidified solution of iron with a solution of potassium thiocyanate in normal butyl alcohol. Normal butyl alcohol was selected over isobutyl and isoamyl alcohols because of its ability to dissolve greater quantities of the thiocyanate. This solvent has a relatively low dielectric constant and the resulting solution of the color complex is stable for several hours.

Flour and bread ash contain small amounts of pyrophosphates, and in the manufacture of enriched and self-rising flours, phosphates, and/or pyrophosphates are often added. On ignition, the phosphates are converted to pyrophosphates which have been shown to interfere seriously with the colorimetric determination of iron, owing to the formation of stable complexes (5, 11, 14, 21). The inhibitory effect of pyrophosphate can be eliminated by employing so-called "wet

TABLE IV  
IRON RECOVERED FROM FLOUR, BREAD, ETC.

|                                           | mg. $P_2O_5$ per g. added<br>as $Na_2HPO_4$ | mg. $P_2O_5$ per g. added<br>as $Na_2P_2O_7$ | mg. $P_2O_5$ per g. added<br>as $CaHPO_4$ | Iron present*<br>μg./g. | Iron added<br>μg./g. | Total iron present<br>μg./g. | Total iron recovered<br>μg./g. | % Recovery |
|-------------------------------------------|---------------------------------------------|----------------------------------------------|-------------------------------------------|-------------------------|----------------------|------------------------------|--------------------------------|------------|
| Unenriched flour                          | —                                           | —                                            | —                                         | 7.9                     | 10.0                 | 17.9                         | 18.1                           | 101.0      |
|                                           | —                                           | —                                            | —                                         | 7.9                     | 10.0                 | 17.9                         | 17.8                           | 99.4       |
|                                           | —                                           | —                                            | —                                         | 7.9                     | 15.0                 | 22.9                         | 23.2                           | 101.3      |
|                                           | —                                           | —                                            | —                                         | 7.9                     | 20.0                 | 27.9                         | 27.4                           | 98.2       |
|                                           | 50                                          | —                                            | —                                         | 7.9                     | 15.0                 | 22.9                         | 22.9                           | 100.0      |
|                                           | 100                                         | —                                            | —                                         | 7.9                     | 15.0                 | 22.9                         | 22.3                           | 97.4       |
|                                           | 25                                          | —                                            | —                                         | 7.9                     | —                    | 7.9                          | 7.8                            | 98.7       |
|                                           | —                                           | 25                                           | —                                         | 7.9                     | —                    | 7.9                          | 7.8                            | 98.7       |
|                                           | —                                           | 25                                           | —                                         | 7.9                     | 15.0                 | 22.9                         | 22.5                           | 98.3       |
|                                           | —                                           | —                                            | 25                                        | 7.9                     | —                    | 7.9                          | 7.7                            | 97.5       |
|                                           | —                                           | —                                            | 25                                        | 7.9                     | 15.0                 | 22.9                         | 23.2                           | 101.3      |
|                                           | —                                           | —                                            | —                                         | 9.8                     | 20.0                 | 29.8                         | 29.4                           | 98.7       |
| Unenriched self-rising flour              | —                                           | —                                            | —                                         | 9.8                     | 20.0                 | 29.8                         | 29.1                           | 97.7       |
|                                           | —                                           | —                                            | —                                         | 9.8                     | 50.0                 | 59.8                         | 58.4                           | 97.7       |
|                                           | —                                           | —                                            | —                                         | 9.8                     | —                    | 30.8                         | 29.4                           | 95.5       |
| Enriched flour                            | 75                                          | —                                            | —                                         | 30.8                    | —                    | 30.8                         | 29.8                           | 96.8       |
|                                           | 25                                          | —                                            | —                                         | 30.8                    | —                    | 30.8                         | 30.0                           | 97.4       |
|                                           | 25                                          | —                                            | —                                         | 30.8                    | —                    | 30.8                         | 30.0                           | 97.4       |
|                                           | —                                           | 25                                           | 30                                        | 30.8                    | —                    | 30.8                         | 30.1                           | 97.7       |
|                                           | —                                           | 50                                           | —                                         | 30.8                    | —                    | 30.8                         | 30.1                           | 97.7       |
| Enriched 100% self-rising flour           | —                                           | 25                                           | —                                         | 30.7                    | —                    | 30.7                         | 30.2                           | 98.4       |
| Enriched 85% extraction self-rising flour | —                                           | —                                            | —                                         | 30.0                    | 10.0                 | 40.0                         | 39.9                           | 99.8       |
|                                           | —                                           | 25                                           | —                                         | 30.0                    | —                    | 30.0                         | 29.4                           | 98.0       |
|                                           | —                                           | 25                                           | —                                         | 30.0                    | 10.0                 | 40.0                         | 39.3                           | 98.3       |
| Enriched 60% extraction self-rising flour | —                                           | —                                            | —                                         | 28.9                    | 10.0                 | 38.9                         | 39.0                           | 100.3      |

\* Determined by this method on materials used prior to addition of  $Na_2HPO_4$  and Fe.

TABLE IV (Continued)

|                                               | mg P <sub>2</sub> O <sub>5</sub> per g added<br>as Na <sub>2</sub> HPO <sub>4</sub> | mg P <sub>2</sub> O <sub>5</sub> per g added<br>as Na <sub>2</sub> P <sub>2</sub> O <sub>7</sub> | mg P <sub>2</sub> O <sub>5</sub> per g added<br>as CaHPO <sub>4</sub> | Iron present*<br>μg/g | Iron added<br>μg/g | Total iron present<br>μg/g | Total iron recovered<br>μg/g | % Recovery |
|-----------------------------------------------|-------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------|-----------------------|--------------------|----------------------------|------------------------------|------------|
| Enriched 40% extraction<br>flour, self-rising | —                                                                                   | 25                                                                                               | —                                                                     | 28.9                  | —                  | 28.9                       | 38.3                         | 97.9       |
|                                               | —                                                                                   | 25                                                                                               | —                                                                     | 28.9                  | 10.0               | 38.9                       | 38.4                         | 98.7       |
|                                               | —                                                                                   | —                                                                                                | —                                                                     | 29.8                  | 10.0               | 39.8                       | 39.7                         | 99.7       |
|                                               | —                                                                                   | 25                                                                                               | —                                                                     | 29.8                  | —                  | 29.8                       | 29.5                         | 98.3       |
| 75% patent enriched phos-<br>phate flour      | —                                                                                   | 25                                                                                               | —                                                                     | 29.8                  | 10.0               | 39.8                       | 39.3                         | 98.7       |
|                                               | —                                                                                   | —                                                                                                | —                                                                     | 31.6                  | 10.0               | 41.6                       | 41.3                         | 99.3       |
|                                               | —                                                                                   | —                                                                                                | —                                                                     | 31.6                  | 20.0               | 51.6                       | 51.1                         | 99.0       |
|                                               | —                                                                                   | 25                                                                                               | —                                                                     | 42.2                  | —                  | 42.2                       | 41.7                         | 98.8       |
| Whole wheat flour                             | —                                                                                   | 25                                                                                               | —                                                                     | 42.2                  | 10.0               | 53.2                       | 52.8                         | 99.2       |
| Rye flour (whole)                             | —                                                                                   | 25                                                                                               | —                                                                     | 11.9                  | —                  | 11.9                       | 11.6                         | 97.5       |
| Enriched white bread                          | —                                                                                   | 25                                                                                               | —                                                                     | 11.9                  | 10.0               | 21.9                       | 21.7                         | 99.1       |
|                                               | —                                                                                   | 25                                                                                               | —                                                                     | 40.8                  | —                  | 40.8                       | 40.2                         | 97.6       |
| Whole wheat bread                             | —                                                                                   | 25                                                                                               | —                                                                     | 40.8                  | 10.0               | 50.8                       | 50.2                         | 98.8       |
|                                               | —                                                                                   | 25                                                                                               | —                                                                     | 51.0                  | —                  | 51.0                       | 50.5                         | 99.0       |
| Bread                                         | —                                                                                   | 25                                                                                               | —                                                                     | 51.0                  | 10.0               | 61.0                       | 50.5                         | 99.2       |
|                                               | —                                                                                   | 25                                                                                               | —                                                                     | 50.8                  | —                  | 50.8                       | 50.4                         | 99.2       |
| Enriched hominy grits                         | —                                                                                   | 25                                                                                               | —                                                                     | 50.8                  | 10.0               | 60.8                       | 60.5                         | 99.5       |
|                                               | —                                                                                   | 25                                                                                               | —                                                                     | 50.8                  | —                  | 50.8                       | 48.9                         | 96.2       |
| Flaked grits                                  | —                                                                                   | 25                                                                                               | —                                                                     | 30.6                  | —                  | 30.6                       | 30.0                         | 98.0       |
|                                               | —                                                                                   | 25                                                                                               | —                                                                     | 30.6                  | 10.0               | 40.6                       | 40.1                         | 98.8       |
| Corn meal                                     | —                                                                                   | —                                                                                                | —                                                                     | 20.5                  | 15.0               | 35.5                       | 34.7                         | 97.7       |
|                                               | —                                                                                   | 25                                                                                               | —                                                                     | 20.5                  | 15.0               | 35.5                       | 34.7                         | 97.7       |
| Malted wheat cereal                           | —                                                                                   | —                                                                                                | —                                                                     | 38.4                  | 15.0               | 53.4                       | 52.8                         | 98.8       |
|                                               | —                                                                                   | 25                                                                                               | —                                                                     | 38.4                  | 15.0               | 53.4                       | 52.3                         | 98.0       |
| Doughnuts                                     | —                                                                                   | —                                                                                                | —                                                                     | 37.7                  | 15.0               | 52.7                       | 51.8                         | 98.3       |
|                                               | —                                                                                   | 25                                                                                               | —                                                                     | 37.7                  | 15.0               | 52.7                       | 51.3                         | 97.3       |
| Doughnut mix                                  | —                                                                                   | —                                                                                                | —                                                                     | 15.2                  | 10.0               | 25.2                       | 25.0                         | 99.3       |
|                                               | —                                                                                   | 25                                                                                               | —                                                                     | 15.2                  | 10.0               | 25.2                       | 24.0                         | 95.2       |
|                                               | —                                                                                   | —                                                                                                | —                                                                     | 16.3                  | 10.0               | 26.3                       | 25.9                         | 98.5       |
|                                               | —                                                                                   | 25                                                                                               | —                                                                     | 16.3                  | 10.0               | 26.3                       | 25.6                         | 97.5       |

ashing" or acid digestion procedures (17), which prevent the formation of pyrophosphate or by hydrolysis of the ash, prior to color development. Hydrolysis can be effected by heating either with acids or alkali (4, 16).

It is to be expected that the interference with the thiocyanate color complex formation, by pyrophosphate, would be decreased by the presence of considerable quantities of any other ion which forms a complex with the pyrophosphate. Monovalent ions have an effect on the inhibiting action of pyrophosphate, divalent ions have an appreciable effect and trivalent positive ions have a very pronounced effect. Aluminum and zirconium have been suggested to liberate iron from its complexes with phosphates (18, 20). Experiments showed

that only 12% of the iron is recoverable directly from 10 ml. of a 1 *N* hydrochloric acid solution, containing 20  $\mu$ g. of iron and 50 mg. of  $P_2O_5$  (added as  $Na_4P_2O_7$ ). Hydrolysis of this solution increased the recovery to 94.5%. The presence of 250 mg. magnesium and hydrolysis increased the experimental recovery to 98.5%. In the presence of 10 mg. of aluminum the experimental recovery was 86.5% and in presence of 100 mg. of aluminum was found to be 98.5%. Table II shows the percentage of iron recovered from solution containing magnesium and aluminum before and after hydrolysis.

It is apparent that smaller amounts of aluminum are as effective as larger amounts of magnesium in overcoming interference of pyrophosphate. Since, in conjunction with hydrolysis, magnesium is just as effective as aluminum, the former was selected for the ashing procedure, because the ash obtained is readily soluble in acid while that of aluminum is practically insoluble.

In Table III are compared a series of samples that have been analyzed by the official A.O.A.C. method and the method herein described. The values obtained by both methods are well within the limits of experimental error.

Analyses carried out on many cereal products are listed in Table IV. In these analyses typical commercial samples were investigated. The initial iron content of the sample was ascertained and in each case additional iron added and the recovery measured. In all instances this was found to be within 2.5% of the theoretical recovery. In some instances, addition of phosphate was also carried out, and in no event did this additional phosphate interfere with complete iron recovery.

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## THE NIACIN AND PANTOTHENIC ACID CONTENT OF CERTAIN OHIO CORN HYBRIDS <sup>1</sup>

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### ABSTRACT

The results of two years' study with eight corn hybrids grown each year in seven different locations in the state of Ohio show that Ohio W36 was significantly higher in niacin than any other corn hybrid studied, while Ohio C38 and Ohio M34 were significantly higher in niacin than Ohio K24, U.S. 13, U.S. 379, and Ohio M20; and Ohio C12 was significantly higher in niacin than U.S. 13, U.S. 379, and Ohio M20; while Ohio K24 was higher in niacin than U.S. 379 and Ohio M20.

The data show that Mahoning County grew corn of the highest niacin content. Paulding, Belmont, and Madison Counties are locations where high niacin corn was produced, but not equal to that of Mahoning, while Hamilton County produced corn of the lowest niacin content.

The results also show that Ohio W36 and Ohio M34 have the highest pantothenic acid content of the eight corn hybrids studied, and U.S. 379 and U.S. 13 contained the lowest amount. The other four corn hybrids are intermediate in pantothenic acid content.

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Pantothenic acid content also appears to be highest in corn hybrids grown in Mahoning County. Belmont County, however, produced corn of a similar (high) pantothenic acid content as that of Mahoning County, while Miami and Madison Counties produced corn of the lowest pantothenic acid content. Hamilton County was also a producer of corn of low pantothenic acid content. The other counties are intermediate between the high and low area producers.

The data show that heredity plays an important role in the niacin and pantothenic acid content of corn and that both vitamins are affected by environmental factors, such as soil and weather, which confirms our previous (Hunt *et al.*, 1947, and Ditzler *et al.*, 1948) observations.

Previous work (Hunt *et al.*, 1947) had shown that the niacin and pantothenic acid content of nine different corn hybrids grown in five corn belt states in three different years varied significantly. In a later study (Ditzler *et al.*, 1948) it was shown that the niacin and pantothenic acid contents were due to the genetic makeup of the hybrids and to the environment (location and season).

The present study was undertaken to obtain further information on niacin and pantothenic acid content of some of the principal double-cross corn hybrids grown in various parts of the state over a period of three years. The 1947 samples were immature, due to late planting and early frost, and therefore the data are not included in this report.

### Materials and Methods

Eight double-cross hybrids grown at seven locations in Ohio during two crop years were included in this study. The corn hybrids studied were Ohio W36, Ohio C38, Ohio M34, Ohio M20, Ohio C12, Ohio K24, U.S. 13, and U.S. 379, and the locations were Mahoning, Madison, Miami, Belmont, Paulding, Wayne, and Hamilton Counties.

The representative samples were obtained by taking the grain from two kernel rows from twelve ears and replicating five times. The final sample was a composite of the above material. The samples were ground to a fine powder and stored in air-tight containers in a dark room. Niacin and pantothenic acid were assayed microbiologically, using methods described in a previous study (Hunt *et al.*, 1947). The niacin and pantothenic acid contents, expressed in  $\mu\text{g. per g.}$ , were calculated to a moisture-free basis. The data for the eight hybrids were subjected to the analysis of variance test to determine the significance of variation due to the genetic makeup of the hybrids, location where grown and the year during which the crop was grown, as well as interactions of these three primary sources of variation.

### Results and Discussion

The niacin and pantothenic acid contents of the corn hybrids are shown in Tables I and II. It is observed that there is little difference

TABLE I  
 NIACIN CONTENT OF OHIO CORN HYBRIDS  
 ( $\mu\text{g. per g.}$ —Moisture-free basis)

| Variety of hybrid | 1945              |          |         |          |       |         |          |         | 1946              |          |         |          |       |         |          |         | Two year average and standard error |
|-------------------|-------------------|----------|---------|----------|-------|---------|----------|---------|-------------------|----------|---------|----------|-------|---------|----------|---------|-------------------------------------|
|                   | Location (county) |          |         |          |       |         |          |         | Location (county) |          |         |          |       |         |          |         |                                     |
|                   | Wayne             | Mahoning | Belmont | Paulding | Miami | Madison | Hamilton | Average | Wayne             | Mahoning | Belmont | Paulding | Miami | Madison | Hamilton | Average |                                     |
| Ohio C12          | 23.1              | 23.1     | 23.1    | 23.3     | 22.4  | 22.0    | 22.4     | 22.8    | 24.4              | 22.2     | 22.4    | 25.3     | 24.1  | 23.0    | 21.0     | 23.2    | 23.0±.3                             |
| Ohio C38          | 21.3              | 26.0     | 26.9    | 24.4     | 20.9  | 24.9    | 20.9     | 23.7    | 22.4              | 21.8     | 25.1    | 27.7     | 23.3  | 24.9    | 20.5     | 23.7    | 23.7±.6                             |
| Ohio K24          | 22.0              | 20.2     | 21.6    | 24.0     | 20.1  | 25.5    | 21.3     | 22.1    | 21.1              | 24.2     | 22.2    | 23.2     | 22.5  | 24.7    | 21.4     | 22.8    | 22.4±.4                             |
| Ohio M20          | 21.9              | 23.0     | 24.2    | 21.9     | 20.2  | 22.8    | 19.8     | 22.0    | 20.1              | 25.7     | 21.3    | 19.8     | 20.7  | 19.7    | 18.1     | 21.0    | 21.5±.5                             |
| Ohio M34          | 24.1              | 21.7     | 24.1    | 26.7     | 24.1  | 26.0    | 23.3     | 24.3    | 20.9              | 28.3     | 22.1    | 23.3     | 22.6  | 22.2    | 21.3     | 23.0    | 23.6±.5                             |
| U. S. 13          | 19.1              | 23.0     | 21.9    | 21.8     | 21.3  | 21.8    | 20.1     | 21.3    | 20.6              | 27.2     | 20.5    | 22.1     | 22.6  | 22.6    | 20.2     | 22.2    | 21.7±.5                             |
| U. S. 379         | 17.0              | 23.9     | 22.1    | 24.5     | 22.1  | 23.1    | 20.4     | 21.9    | 20.8              | 25.4     | 20.4    | 21.2     | 21.1  | 19.8    | 17.7     | 20.9    | 21.4±.6                             |
| Ohio W36          | 24.9              | 27.2     | 30.1    | 29.5     | 24.3  | 25.9    | 24.9     | 26.6    | 24.7              | 24.0     | 22.7    | 25.9     | 24.5  | 24.2    | 23.0     | 24.1    | 25.3±.5                             |
| Average           | 21.7              | 23.7     | 24.7    | 24.9     | 22.1  | 24.4    | 21.7     | 23.3    | 21.9              | 25.0     | 22.2    | 23.7     | 22.5  | 21.9    | 20.3     | 22.5    | 22.5±.2                             |
| Two-year average  | 21.8              | 24.3     | 23.5    | 24.3     | 22.3  | 23.1    | 21.0     |         |                   |          |         |          |       |         |          |         |                                     |

Minimum significant difference (amount by which a pair of two-year averages must differ to be significant from each other).  
 Location, 0.8, significant; 1.0, highly significant.  
 Hybrid, 0.8, significant; 1.1, highly significant.

TABLE II  
PANTOTHENIC ACID CONTENT OF OHIO CORN HYBRIDS  
( $\mu\text{g. per g.}$ —Moisture-free basis)

| Variety of hybrid | 1945              |           |         |          |       |         |          |         | 1946              |           |         |          |       |         |          |         | Two year average and standard error |
|-------------------|-------------------|-----------|---------|----------|-------|---------|----------|---------|-------------------|-----------|---------|----------|-------|---------|----------|---------|-------------------------------------|
|                   | Location (county) |           |         |          |       |         |          |         | Location (county) |           |         |          |       |         |          |         |                                     |
|                   | Wayne             | Malhoning | Belmont | Paulding | Miami | Madison | Hamilton | Average | Wayne             | Malhoning | Belmont | Paulding | Miami | Madison | Hamilton | Average |                                     |
| Ohio C12          | 4.8               | 4.2       | 6.1     | 3.7      | 3.3   | 3.2     | 4.2      | 4.2     | 3.7               | 6.2       | 3.9     | 6.2      | 3.0   | 2.8     | 3.0      | 4.1     |                                     |
| Ohio C38          | 5.2               | 3.7       | 6.7     | 4.1      | 3.4   | 3.6     | 4.3      | 4.4     | 3.6               | 4.7       | 2.7     | 5.6      | 3.8   | 4.7     | 3.9      | 4.2     |                                     |
| Ohio K24          | 4.2               | 3.2       | 6.8     | 3.9      | 4.9   | 3.3     | 4.9      | 4.4     | 4.0               | 5.2       | 2.8     | 4.8      | 4.2   | 5.3     | 4.9      | 4.4     |                                     |
| Ohio M20          | 3.8               | 3.9       | 5.9     | 3.6      | 3.2   | 3.4     | 3.7      | 3.9     | 3.8               | 6.1       | 3.4     | 4.7      | 5.6   | 4.6     | 4.1      | 4.6     |                                     |
| Ohio M34          | 4.3               | 4.0       | 6.3     | 4.9      | 3.7   | 4.1     | 5.1      | 4.7     | 4.6               | 7.2       | 3.9     | 5.6      | 4.2   | 4.9     | 4.9      | 5.0     |                                     |
| U. S. 13          | 4.6               | 4.0       | 4.1     | 2.3      | 2.7   | 2.1     | 2.6      | 3.2     | 4.0               | 6.1       | 2.9     | 4.6      | 3.8   | 4.2     | 4.2      | 4.2     |                                     |
| U. S. 379         | 4.0               | 4.1       | 8.6     | 3.2      | 2.4   | 1.9     | 3.0      | 3.9     | 3.1               | 5.4       | 2.9     | 4.9      | 3.4   | 4.2     | 2.6      | 3.8     |                                     |
| Ohio W36          | 5.1               | 2.8       | 7.2     | 3.3      | 3.0   | 4.7     | 4.6      | 4.3     | 5.6               | 8.6       | 3.7     | 6.3      | 4.4   | 5.9     | 3.7      | 5.4     |                                     |
| Average           | 4.6               | 3.7       | 6.4     | 3.8      | 3.2   | 3.2     | 4.0      | 4.1     | 4.0               | 6.1       | 3.2     | 5.1      | 4.0   | 4.4     | 3.8      | 4.4     |                                     |
| Two-year average  | 4.3               | 4.9       | 4.8     | 4.4      | 3.6   | 3.8     | 3.9      | 4.3     |                   |           |         |          |       |         |          |         |                                     |

Minimum significant difference (amount by which a pair of two-year averages must differ to be significant from each other):

Location, 0.5, significant; 0.7, highly significant.

Hybrid, 0.5, significant; 0.6, highly significant.

TABLE III  
THE NIACIN AND PANTOTHENIC ACID CONTENT OF CORN INBREDS (PARENTS OF CORN HYBRIDS)  
( $\mu\text{g. per g.}$ —Moisture-free basis)

| Hybrid    | Genetic makeup (inbred lines)  | Niacin             | Av.  | Pantothenic acid | Av. |
|-----------|--------------------------------|--------------------|------|------------------|-----|
| Ohio C12  | (Ind. WF9 $\times$ Oh. 07)     | 25.8 $\times$ 27.0 | 20.2 | 8.2 $\times$ 5.8 | 5.3 |
| Ohio C38  | (Ind. WF9 $\times$ Hy)         | 25.8 $\times$ 15.8 | 21.8 | 8.2 $\times$ 3.3 | 6.4 |
| Ohio K24  | (Oh. 51A $\times$ WF9)         | 23.0 $\times$ 25.8 | 18.9 | 5.5 $\times$ 8.2 | 6.4 |
| Ohio M20  | (Oh. 26 $\times$ Oh. 51)       | 15.8 $\times$ 16.8 | 14.9 | 6.6 $\times$ 6.2 | 6.2 |
| Ohio M34  | (Oh. 26 $\times$ Oh. 51)       | 15.8 $\times$ 16.8 | 19.5 | 6.6 $\times$ 6.2 | 6.7 |
| U. S. 13  | (Ill. Hy $\times$ Ia. L317)    | 15.8 $\times$ 12.4 | 18.6 | 3.3 $\times$ 4.1 | 5.4 |
| U. S. 379 | (Ill. Hy $\times$ C17)         | 15.8 $\times$ 24.7 | 19.8 | 3.3 $\times$ 8.1 | 5.8 |
| Ohio W36  | (Oh. 51A $\times$ Ind. WF9)    | 23.0 $\times$ 25.8 | 23.6 | 5.5 $\times$ 8.2 | 7.0 |
|           | (Oh. 40B $\times$ Oh. 02)      | 13.6 $\times$ 32.0 |      | 7.3 $\times$ 6.9 |     |
|           | (Ill. Hy $\times$ Ia. L317)    | 15.8 $\times$ 12.4 |      | 3.3 $\times$ 4.1 |     |
|           | (Oh. 40B $\times$ Oh. 02)      | 13.3 $\times$ 13.6 |      | 4.6 $\times$ 7.3 |     |
|           | (Oh. 33 $\times$ Oh. 40B)      | 13.3 $\times$ 13.6 |      | 4.6 $\times$ 7.3 |     |
|           | (Oh. 40B $\times$ Oh. 02)      | 13.6 $\times$ 32.0 |      | 7.3 $\times$ 6.9 |     |
|           | (Ind. WF9 $\times$ Ind. 38-11) | 25.8 $\times$ 20.6 |      | 8.2 $\times$ 6.2 |     |
|           | (Ia. P8 $\times$ T8)           | 17.8 $\times$ 19.1 |      | 5.8 $\times$ 6.1 |     |
|           | (Oh. 40B $\times$ Oh. 02)      | 13.6 $\times$ 32.0 |      | 7.3 $\times$ 6.9 |     |



in the average niacin and pantothenic acid content for each corn hybrid from all locations due to year, but there is considerable difference in the vitamin content of each hybrid from one location to another. This shows that these two vitamins can be influenced by soil and other environmental factors. These differences are greater, on a percentage basis, in the case of pantothenic acid than in the case of niacin.

For comparative purposes, the genetic makeup of the corn hybrids with niacin and pantothenic acid content of each, and the inbreds<sup>3</sup> of which each hybrid is compared, are given in Table III.

The results of statistical analysis show that Ohio W36 is significantly higher in niacin than all other hybrids used in this study. In comparison of Ohio W36 with Ohio C38 and Ohio K24, the only difference in their genetic makeup is a single inbred; Ohio W36 having three high niacin inbred lines while Ohio C38 and Ohio K24 have only two high niacin inbred lines. Hybrid Ohio M20 has four inbred lines

TABLE IV  
VARIANCES FOR NIACIN AND PANTOTHENIC ACID CONTENTS  
OF CORN HYBRIDS

| Source of variance | Degrees of freedom | Niacin  | Pantothenic acid |
|--------------------|--------------------|---------|------------------|
| Location           | 6                  | 17.76** | 3.05**           |
| Hybrid             | 7                  | 21.77** | 2.02**           |
| Year               | 1                  | 5.45*   | 2.49*            |
| Location×hybrid    | 42                 | 2.12*   | .45              |
| Location×year      | 6                  | 11.29** | 11.30**          |
| Hybrid×year        | 7                  | 4.15**  | .87              |
| Error              | 42                 | 1.06    | 0.39             |
| Total              | 111                |         |                  |

\* Significant. \*\* Highly significant.

of low niacin content in its genetic makeup and it was the lowest in niacin content of any one of the corn hybrids studied, with the possible exception of U.S. 379; while Ohio M34 has only three inbred lines of low niacin content and it was significantly higher in niacin than Ohio M20. In a similar way, it can be shown why Ohio C38 and Ohio M34 have a significantly higher niacin content than U.S. 13 and possibly U.S. 379. Unfortunately, the inbreds of which the corn hybrids are composed were not grown the same year or years, nor in the same location as the hybrids under study, and as a consequence the results (genetically speaking) may not appear to present as clear a relationship as might be expected. This is no doubt due to difference in the environmental factor or factors to which the hybrids and inbreds were

<sup>3</sup> Lorraine Rodriguez, Chas. H. Hunt, and R. M. Bethke. Protein, niacin, and pantothenic acid in corn inbred lines. In print.

TABLE V  
EFFECT OF LOCATION ON THE NIACIN CONTENT OF CORN HYBRIDS

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|                                                                                                   |
|---------------------------------------------------------------------------------------------------|
| Mahoning County, higher niacin content than Madison,* Belmont,* Miami,** Wayne,** and Hamilton.** |
| Paulding County, higher niacin content than Miami,** Wayne,** and Hamilton.**                     |
| Madison County, higher niacin content than Wayne** and Belmont.**                                 |
| Belmont County, higher niacin content than Miami,* Wayne,** and Hamilton.**                       |
| Miami County, higher niacin content than Hamilton.**                                              |

---

\* Significant    \*\* Highly significant.

exposed. However, it is interesting to note that the decreasing order of the niacin values of the corn hybrids is in the same position, with one exception, as the calculated values (average niacin content of inbreds for each hybrid). See Tables I and III.

As previously stated, there was considerable variation in the niacin content of each hybrid from location to location. The variances due to location, hybrid, year, and the various interactions are shown in Table IV. The results of the analysis of variance due to location are shown in Table V.

A statistical analysis of the data (Table II) shows that Ohio W36 and Ohio M34 are significantly higher in pantothenic acid than Ohio C38, Ohio M20, Ohio C12, U.S. 379, and U.S. 13, while Ohio K24 is higher than U.S. 379 and U.S. 13. Also, Ohio C38 is higher in pantothenic acid than U.S. 13. Furthermore, Ohio M34 is significantly higher in pantothenic acid than Ohio M20. Both have the same genetic parents, with one exception. Ohio M34 has as one parent inbred (Oh 02), with a pantothenic acid content of 6.2  $\mu\text{g. per g.}$ , while Ohio M20 has as one parent inbred (Oh 33), with a pantothenic acid content of 4.1  $\mu\text{g. per g.}$  This clearly demonstrates the genetic influence on the hybrids themselves. This is again substantiated by the fact that the decreasing order of the pantothenic acid values of the corn hybrids under study is, with two exceptions, in the same position as the calculated values (see Tables II and III).

TABLE VI  
EFFECT OF LOCATION ON THE PANTOTHENIC ACID CONTENT  
OF CORN HYBRIDS

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|                                                                                                               |
|---------------------------------------------------------------------------------------------------------------|
| Mahoning County, higher pantothenic acid content than Paulding,* Wayne,** Hamilton,** Madison,** and Miami.** |
| Belmont County, higher pantothenic acid content than Paulding,* Wayne,** Hamilton,** Madison,** and Miami.**  |
| Paulding County, higher pantothenic acid content than Hamilton,* Madison,* and Miami.**                       |
| Wayne County, higher pantothenic content than Miami.*                                                         |

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\* Significant.    \*\* Highly significant.

The calculated values as one observes are higher than the actual values. This apparent discrepancy may be due to the difference in the environmental factors and the fact that the inbreds and hybrids were not taken from the same location and in the same year.

The results of the analysis of variance due to location are shown in Table VI.

The survey shows that corn hybrid Ohio W36 has the highest niacin content and, with one exception, the highest pantothenic acid content, while Ohio M20, U.S. 13, and U.S. 379 are the lowest in niacin, and U.S. 13 is the lowest in pantothenic acid.

The location effect shows that Mahoning County produced corn of the highest niacin and pantothenic acid content, while Hamilton County grew corn of the lowest niacin content, and Miami County, the lowest pantothenic acid content.

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# THE EFFECT OF MATURITY ON THE NIACIN AND PANTOTHENIC ACID CONTENT OF THE STALKS AND LEAVES, TASSELS, AND GRAIN OF FOUR SWEET CORN VARIETIES<sup>1</sup>

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## ABSTRACT

Niacin and pantothenic acid and percent dry matter were determined in the stalks and leaves, tassels, and grain of four varieties of sweet corn sampled at various stages of maturity. The dry matter and niacin content of stalks and leaves remained remarkably constant for the first six periods of collection or until the ears were well filled. The pantothenic acid content varied irregularly.

The niacin content of the tassels decreased regularly after pollination, with the highest value at that time. The pantothenic acid content of the tassel decreased as the plant matured but the decrease was not so regular as that of niacin. The niacin content of the pollen collected at bloom stage was higher than that of the tassel.

The dry matter of the grain increased two-fold over the period of collection and at the same time the niacin and pantothenic acid content decreased as the grain matured, but not proportionately to the increase in dry matter. The highest concentration of niacin and pantothenic acid in the grain occurred 18 to 20 days after the tassel bloomed. This is the "roasting-ear stage" when the corn should be consumed.

The possibility of developing high-niacin corn by breeding has been shown by several investigators (1, 2, 3, 4, 5, 6, 7). Hunt *et al.* (1947) and Ditzler *et al.* (1948) have also shown that pantothenic acid is regulated by inheritance and that the niacin and pantothenic acid contents of corn hybrids are influenced to some extent by such environmental factors as season and location.

The present investigations were undertaken to obtain information on the niacin and pantothenic acid content of the grain and other parts of the sweet corn plant at various stages of maturity, and to show the relation between the vitamin contents of the different parts of the plant. Earlier studies had shown that maturity affected the niacin and pantothenic acid content of field corn (unpublished data).

## Materials and Methods

The varieties of sweet corn used were Marcross, Ohiogold, Golden Cross Bantam, and Stowell's Evergreen; all grown in the same field at

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Wooster in 1948. Marcross is an early-maturing variety, Ohiogold and Golden Cross Bantam are intermediate, and Stowell's Evergreen is a late-maturing variety. Five replications of each variety were grown under similar environmental conditions.

The sampling procedure in the field was as follows: Samples were collected at seven to eleven-day intervals from July 19 to September 14, and again on October 21. The stalks and leaves were collected throughout the period, tassels as soon as they appeared, and grain as soon as it was developed sufficiently for sampling. One pollen sample from each variety was collected when the tassels were in full or near full bloom.

The stalks were cut at the first or second node above the ear, or, at the early dates, at the place where the ear was appearing. This was partly determined by the maturity of the plant on the sampling date. The stalks and leaves were gathered as one sample, and the tassels and grain as separate samples. Duplicate samples of leaves and stalks, tassels, and grain were gathered which were composites of the five replications. These are designated as samples A and B.

Immediately after gathering, the stalk and leaves and tassel samples were chopped in a Hobart mill to form a mixture, after which a 100 g. portion was removed for moisture determinations. The remaining part of the sample was run through a Waring Blendor and stored in a paraffined carton at freezing temperature (15°F.) until assayed.

The grains were cut from the cob and the cob was scraped to remove all of the grain. The grain was then mixed in a Waring Blendor, a portion removed for moisture determination, and the remainder stored in paraffined cartons at freezing temperature.

The fresh or frozen samples were assayed microbiologically for niacin and pantothenic acid, according to methods described in a previous study (Hunt *et al.*, 1947). The assay values were calculated to a dry matter basis.

### Results and Discussion

The niacin and pantothenic acid contents as well as the percentage dry matter of the various parts of the sweet corn plant, at different stages of maturity, are shown in Table 1. The data given are averages of samples A and B.

Marcross was the most mature of the four varieties at the date of the first collection, and it matured more rapidly than the other three varieties. For this reason, samples collected at seven to eleven day intervals made a less complete record of the variation due to maturity in Marcross than in the other varieties.

The dry matter of the stalks and leaves of Marcross increased from 15.0 to 20.5% from July 19 to August 23; the niacin content increased,

irregularly, from 44.0 to 54.0  $\mu\text{g.}$ , and the pantothenic acid content increased from 11.7 to 18.9  $\mu\text{g. per g.}$  in the same period. The dry matter of the tassels of Marcross increased from 29.2 to 42.0% between July 19 and August 3, then decreased to 37.3% by August 23; while the niacin content of the tassels fell from 45.0 to 33.3  $\mu\text{g.}$  between July 19 and July 27 and remained more or less constant thereafter. The pantothenic acid content of the same variety showed similar behavior, making its greatest decrease (37.1 to 23.0  $\mu\text{g.}$ ) from July 19 to July 27. The dry matter of the grain of Marcross increased from 18.1 to 36.2%, a two-fold increase, from August 3 to August 23. The niacin content decreased from 61.7 to 51.5  $\mu\text{g.}$  from August 3 to August 23, while the pantothenic acid content showed little variation for the same period.

The variation in the niacin and pantothenic acid content of maturing Golden Cross Bantam and Ohiogold were generally similar. Stowell's Evergreen, a late-maturing variety, exhibited the same variation a few days later than the other two.

The dry matter of stalks and leaves of all three varieties (Golden Cross Bantam, Ohiogold, and Stowell's Evergreen) increased (15.3 to 26.2, 17.0 to 27.5, and 16.4 to 26.2%, respectively) from July 19 to September 14.

The niacin content of stalks and leaves of Golden Cross Bantam, Ohiogold, and Stowell's Evergreen remained relatively constant until the last two collection dates (September 7 and 14), when there was a marked drop. The pantothenic acid content of the stalks and leaves of all three varieties (Golden Cross Bantam, Ohiogold, and Stowell's Evergreen) varied irregularly, being lowest at the middle collection period, with the highest values at the beginning and end of the collections.

The dry matter of the tassels of all three varieties (Golden Cross Bantam, Ohiogold, and Stowell's Evergreen) increased considerably (22.0 to 60.6, 24.2 to 61.4, and 21.0 to 58.9%, respectively). The niacin content of the tassels of each corn decreased regularly, with the highest values at the first or second collection periods. The pantothenic acid content of the tassels varied irregularly, with a general downward trend.

The dry matter of the grain of all three varieties (Golden Cross Bantam, Ohiogold, and Stowell's Evergreen) showed a two-fold increase over the period of collection (22.2 to 46.4, 18.0 to 41.8, and 16.6 to 33.6%, respectively). The niacin and pantothenic acid contents of the grain decreased as the grain matured but not proportionately to the increase in dry matter. Variations existed among the varieties as to the amounts of both vitamins present at any given stage of maturity.

TABLE I

EFFECT OF MATURITY ON THE NIACIN AND PANTOTHENIC ACID CONTENT  
OF THE VARIOUS PARTS OF THE CORN PLANT.  
MICROGRAMS PER GRAM. MOISTURE FREE

| Date of harvest     | Stalks and leaves |        |                  | Tassels      |        |                  | Grain        |        |                  | Pollen  |                  |
|---------------------|-------------------|--------|------------------|--------------|--------|------------------|--------------|--------|------------------|---------|------------------|
|                     | Dry matter %      | Niacin | Pantothenic acid | Dry matter % | Niacin | Pantothenic acid | Dry matter % | Niacin | Pantothenic acid | Niacin  | Pantothenic acid |
| MARCROSS            |                   |        |                  |              |        |                  |              |        |                  |         |                  |
| Jul. 19             | 15.0              | 44.0   | 11.7             | 29.2         | 45.0   | 37.0             |              |        |                  | July 24 |                  |
| Jul. 27             | 18.9              | 52.7   | 10.5             | 36.8         | 33.0   | 23.0             |              |        |                  | 65.9    | 13.0             |
| Aug. 3              | 18.9              | 53.7   | 13.5             | 42.0         | 31.7   | 24.0             | 18.1         | 61.7   | 17.0             |         |                  |
| Aug. 12             | 20.5              | 46.8   | 14.8             | 39.6         | 27.8   | 23.5             | 23.9         | 61.9   | 20.4             |         |                  |
| Aug. 23             | 19.1              | 54.0   | 18.9             | 37.3         | 32.0   | 21.8             | 36.2         | 51.5   | 16.6             |         |                  |
| Oct. 21             |                   | 9.7    |                  |              |        |                  |              | 26.0   | 8.0              |         |                  |
| GOLDEN CROSS BANTAM |                   |        |                  |              |        |                  |              |        |                  |         |                  |
| Jul. 19             | 15.3              | 41.0   | 13.0             |              |        |                  |              |        |                  | 69.0    |                  |
| Jul. 27             | 16.9              | 43.9   | 10.0             | 22.0         | 53.4   | 28.4             |              |        |                  |         |                  |
| Aug. 3              | 17.2              | 43.0   | 7.0              | 32.2         | 59.5   | 30.0             |              |        |                  |         |                  |
| Aug. 12             | 19.4              | 40.8   | 9.9              | 36.3         | 39.0   | 29.7             |              |        |                  |         |                  |
| Aug. 23             | 20.5              | 45.4   | 11.0             | 35.2         | 38.6   | 17.0             | 22.2         | 81.4   | 37.5             |         |                  |
| Aug. 31             | 22.4              | 44.0   | 17.9             | 41.6         | 42.0   | 33.0             | 34.0         | 47.6   | 25.5             |         |                  |
| Sept. 7             | 24.5              | 34.7   | 11.5             | 57.0         | 27.7   | 18.4             | 38.0         | 40.4   | 17.0             |         |                  |
| Sept. 14            | 26.2              | 34.0   | 23.0             | 60.6         | 24.0   | 12.7             | 46.4         | 34.1   | 12.5             |         |                  |
| Oct. 21             |                   | 13.7   |                  |              |        |                  |              | 23.3   | 7.0              |         |                  |
| OHIOGOLD            |                   |        |                  |              |        |                  |              |        |                  |         |                  |
| Jul. 19             | 17.0              | 39.7   | 13.0             |              |        |                  |              |        |                  | 81.5    | 7.6              |
| Jul. 27             | 17.4              | 48.0   | 11.0             | 24.2         | 49.4   | 23.0             |              |        |                  |         |                  |
| Aug. 3              | 19.1              | 45.5   | 6.0              | 35.6         | 52.8   | 27.6             |              |        |                  |         |                  |
| Aug. 12             | 20.6              | 42.0   | 8.5              | 36.0         | 46.7   | 27.5             |              |        |                  |         |                  |
| Aug. 23             | 23.0              | 40.7   | 9.8              | 38.2         | 35.0   | 18.0             | 18.0         | 94.1   | 40.0             |         |                  |
| Aug. 31             | 23.2              | 44.6   | 13.8             | 41.6         | 37.6   | 28.0             | 30.6         | 59.2   | 30.6             |         |                  |
| Sept. 7             | 26.5              | 34.0   | 7.0              | 52.0         | 31.5   | 18.0             | 34.3         | 60.6   | 17.0             |         |                  |
| Sept. 14            | 27.5              | 36.7   | 18.5             | 61.4         | 25.0   | 19.7             | 41.8         | 49.3   | 13.0             |         |                  |
| Oct. 21             |                   | 16.0   |                  |              |        |                  |              | 37.0   | 7.5              |         |                  |
| STOWELL'S EVERGREEN |                   |        |                  |              |        |                  |              |        |                  |         |                  |
| Jul. 19             | 16.4              | 42.0   | 12.0             |              |        |                  |              |        |                  | 56.0    | 13.5             |
| Jul. 27             | 17.4              | 46.9   | 10.0             | 21.0         | 58.8   | 26.0             |              |        |                  |         |                  |
| Aug. 3              | 18.6              | 51.0   | 6.6              | 32.8         | 53.7   | 23.6             |              |        |                  |         |                  |
| Aug. 12             | 19.5              | 44.4   | 9.6              | 32.8         | 46.0   | 24.9             |              |        |                  |         |                  |
| Aug. 23             | 23.3              | 37.8   | 8.0              | 37.6         | 38.7   | 13.0             | 16.6         | 80.8   | 30.0             |         |                  |
| Aug. 31             | 22.9              | 41.8   | 9.6              | 45.2         | 39.0   | 22.0             | 24.1         | 94.4   | 27.4             |         |                  |
| Sept. 7             | 26.0              | 35.8   | 7.6              | 52.0         | 30.0   | 12.0             | 27.8         | 80.4   | 19.6             |         |                  |
| Sept. 14            | 26.2              | 36.6   | 12.0             | 58.9         | 28.7   | 18.0             | 33.6         | 62.5   | 18.0             |         |                  |
| Oct. 21             |                   | 13.0   |                  |              |        |                  |              | 48.7   | 3.0              |         |                  |

The vitamin contents of both stalks and leaves and grain of all varieties collected on October 21 demonstrate that the downward trend continued after the regular collections were stopped. The varietal variations evident earlier in the vitamin contents of grain continued, and varietal variations in vitamin content of dry stalk and leaf tissue became evident.

As noted earlier, the vitamin content of the stalks and leaves of all varieties remained relatively constant until the ear was well formed. This may be attributable to the fact that the primary function of the leaf is to synthesize carbohydrates, which are then translocated to the ear. It may be that a similar synthesis and translocation can be said to occur in the case of niacin and pantothenic acid.

In the tassels, the highest concentration of the vitamins, particularly niacin, occurred just before or during the dispersion of the pollen "bloom stage." The niacin content of the pollen was higher than that of the tassel at this stage. The same was not true of pantothenic acid.

The highest concentration of both vitamins in the grain occurred eighteen to twenty days after the "bloom" stage, or when the pollen was dispersed. Corn consumed at this stage is succulent and sweet and is capable of adding considerable niacin and pantothenic acid to the human dietary.

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# THE QUALITY OF HARD RED WINTER WHEAT AS AFFECTED BY 2,4-D SPRAY APPLICATIONS<sup>1,2</sup>

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## ABSTRACT

Samples of hard red winter wheat grown in 1948 at the Fort Hays (Kansas) Branch Experiment Station and representing various dates, types, and dosages of 2,4-D application revealed no reduction in wheat yield resulting from the treatment with 2,4-D. Only one date of application of 2,4-D in four produced a significant increase in wheat protein. When yield of protein (per cent protein  $\times$  yield) was considered there was no difference between treated and untreated samples. Applications of 2,4-D did not affect the mineral content, milling, or baking quality of the wheats. These conclusions were based on one year's samples, obtained from one location.

Erickson, Seely, and Klages (2) and Sibbitt and Harris (7) have reported that certain applications of 2,4-D treatments on weed-free plots increased the protein content of the kernels. Sibbitt and Harris (7) have also reported that applications of the acetate, the triethyl amine, and the butyl ester of 2,4-dichlorophenoxyacetic acid to wheat plants at different stages of growth did not impair the milling or baking quality of the wheat. Several varieties of both spring and winter wheat were investigated (2, 7) and at the Idaho Experiment Station (2) the trials were conducted on both dry land and irrigated plots.

Klingman (4) and McNeal (5) have shown that wheat yields are usually reduced significantly by 2,4-D treatments. Since previous workers have not reported on the protein content of wheat as related to yield, it seemed desirable to study the effect of 2,4-D treatments on the protein content of wheat when yield of protein (protein  $\times$  yield) is considered. The effect of 2,4-D treatments on the mineral content of wheat was also investigated.

## Materials and Methods

The wheat for this study was grown at the Fort Hays Branch Experiment Station in Central Kansas and harvested in 1948. The wheat was normal as indicated by many comparative analyses and milling and baking tests (6).

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<sup>2</sup> Cooperative investigation between the Division of Cereal Crops and Diseases, Bureau of Plant Industry, Soils and Agricultural Engineering, U. S. Department of Agriculture and the Kansas Agricultural Experiment Station; Contribution No. 171, Department of Milling Industry, and Contribution No. 54, Fort Hays Experiment Station.

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<sup>4</sup> Assistant agronomist and associate chemist, respectively, Division of Cereal Crops and Diseases.

The date and type of treatment on the randomized plots, replicated three times, are shown in Table I. Methods of application included both ground and airplane spraying; however, in the interest of shortening and simplifying this presentation, only the ground spraying experiments are included. The results were essentially the same in both experiments. One hundred and thirty-five samples representing various dates, types, and dosages of 2,4-D applications were harvested. All the wheat plots, including the check plots, were hand-weeded. Moisture, protein, and ash determinations were made on all samples using the methods outlined in *Cereal Laboratory Methods*, 5th ed. (1).

The baking tests were conducted using a standard sponge formula and baking procedure as described by Johnson and Miller (3). A total of 700 g. of flour was mixed in a Hobart A-200 mixer. Two 250-g. doughs were divided and baked from each mix.

### Results and Discussion

Table I summarizes the yield, protein, and ash determinations for the wheat samples subjected to various 2,4-D treatments at different stages of growth.

A summary of the analysis of variance for ash, protein, and protein  $\times$  yield data is presented in Table II. Variation between groups was significant for ash, protein, and yield, but not for yield of protein. Replications within groups were also significant for all of the factors studied. This can possibly be explained by the wide distribution of the plots, although the entire experiment was conducted in the same area on what appeared to be uniform soil conditions.

*Yield.* The various 2,4-D treatments did not reduce the yield of wheat significantly (Table II). This is not in agreement with the work of Klingman (4) or McNeal (5). However, the evidence presented here indicates that the trend in treated plots as compared with untreated plots is toward a reduction in yield of wheat.

*Protein.* Examination of Table I shows that there is no consistent change in protein content with increased dosages of the various treatments. In most instances, however, the protein content of the wheat tended to increase by 2,4-D applications as compared to the untreated plots. Group C was the only group which showed significant treatment differences with respect to per cent protein. The grain in this group was in the heading stage during the application of the spray. Rainfall following this application amounted to 4.27 in. during the next 14 days and temperatures were relatively low during this period. In contrast only 1.23 in. of rain were received in the two weeks following the April 23 treatment and none from May 11 to May 29.

TABLE I  
EFFECT OF VARIOUS 2,4-D SURFACE SPRAY APPLICATIONS ON THE YIELD,  
PROTEIN, AND ASH OF HARD RED WINTER WHEAT

| Formulation                                                          | Mean values |                      |                  |
|----------------------------------------------------------------------|-------------|----------------------|------------------|
|                                                                      | Yield       | Protein <sup>1</sup> | Ash <sup>1</sup> |
|                                                                      | bu./A.      | %                    | %                |
| GROUP A                                                              |             |                      |                  |
| Date of treatment—April 26, 1949; stage of growth—pre-fully tillered |             |                      |                  |
| None                                                                 | 50.8        | 14.6                 | 1.69             |
| Sodium salt <sup>2</sup>                                             | 50.0        | 14.5                 | 1.75             |
| Butyl ester <sup>3</sup>                                             | 45.5        | 14.7                 | 1.71             |
| Butyl ester + diesel oil <sup>1</sup>                                | 50.1        | 14.5                 | 1.73             |
| Average difference due to treatment                                  | - 2.3       | 0.0                  | +0.04            |
| GROUP B                                                              |             |                      |                  |
| Date of treatment—May 11, 1949; stage of growth—second joint         |             |                      |                  |
| None                                                                 | 47.6        | 14.5                 | 1.71             |
| Sodium salt <sup>5</sup>                                             | 48.0        | 14.8                 | 1.73             |
| Butyl ester <sup>6</sup>                                             | 44.5        | 14.7                 | 1.78             |
| Butyl ester + diesel oil <sup>7</sup>                                | 46.0        | 14.6                 | 1.81             |
| Average difference due to treatment                                  | - 1.4       | + 0.2                | +0.06            |
| GROUP C                                                              |             |                      |                  |
| Date of treatment—May 27, 1949; stage of growth—early heading        |             |                      |                  |
| None                                                                 | 41.1        | 14.7                 | 1.63             |
| Sodium salt <sup>8</sup>                                             | 43.0        | 14.7                 | 1.69             |
| Butyl ester <sup>9</sup>                                             | 39.0        | 15.2                 | 1.68             |
| Butyl ester + diesel oil <sup>4</sup>                                | 38.5        | 15.1                 | 1.68             |
| Average difference due to treatment                                  | - 0.9       | + 0.3                | +0.05            |
| GROUP D                                                              |             |                      |                  |
| Date of treatment—June 23, 1949; stage of growth—late dough          |             |                      |                  |
| None                                                                 | 44.0        | 14.5                 | 1.66             |
| Sodium salt <sup>2</sup>                                             | 42.3        | 14.8                 | 1.66             |
| Butyl ester <sup>3</sup>                                             | 45.0        | 14.6                 | 1.66             |
| Butyl ester + diesel oil <sup>4</sup>                                | 44.2        | 14.8                 | 1.62             |
| Average difference due to treatment                                  | - 0.2       | + 0.2                | -0.01            |

<sup>1</sup> Results reported on 14% moisture basis.

<sup>2</sup> Mean of results from triplicated plots to which 0.25, 0.5, and 1.0 lb. quantities of the sodium salt of 2,4-dichlorophenoxyacetic acid were added as a surface spray.

<sup>3</sup> Same as footnote 2, except the butyl ester of 2,4-dichlorophenoxyacetic acid was employed.

<sup>4</sup> Same as footnote 2, except the butyl ester of 2,4-dichlorophenoxyacetic acid in 5 qts. of diesel oil was employed.

<sup>5</sup> Same as footnote 2, except 0.25, 0.5, 1.0, and 1.5 lb. quantities of the sodium salt of 2,4-dichlorophenoxyacetic acid were employed.

<sup>6</sup> Same as footnote 2, except 0.12, 0.25, 0.5, 0.75, and 1.0 lb. quantities of the butyl ester of 2,4-dichlorophenoxyacetic acid were employed.

<sup>7</sup> Same as footnote 2, except 0.12, 0.25, 0.5, 0.75, and 1.0 lb. quantities of the butyl ester of 2,4-dichlorophenoxyacetic acid in 5 qts. of diesel oil were employed.

Average protein differences were sufficiently large to indicate a significant treatment effect in the analysis of the data from all groups (Table II). Least significant mean differences determined in the usual way showed that the mean value of per cent protein for Group C was significantly higher than that for any of the other groups. However, when total protein (per cent protein  $\times$  yield) data were

TABLE II  
EFFECT OF 2,4-D TREATMENTS ON WHEAT YIELD, PROTEIN, ASH, AND YIELD OF PROTEIN (PROTEIN  $\times$  YIELD)

| Sources of variation                                                 | Degrees of freedom | Mean squares yield | Mean squares protein | Mean squares ash | Mean squares protein $\times$ yield |
|----------------------------------------------------------------------|--------------------|--------------------|----------------------|------------------|-------------------------------------|
| ANALYSES OF VARIANCE BY GROUPS                                       |                    |                    |                      |                  |                                     |
| Group A—Treatment April 26, 1948—Stage of growth, pre-fully tillered |                    |                    |                      |                  |                                     |
| Replications                                                         | 2                  | 143.4**            | 0.36**               | 0.009            | 2.18*                               |
| Treatments                                                           | 9                  | 19.6               | 0.02                 | 0.003            | 0.33                                |
| Error                                                                | 17                 | 23.4               | 0.04                 | 0.003            | 0.48                                |
| Total                                                                | 28                 |                    |                      |                  |                                     |
| Group B—Treatment May 11, 1948—Stage of growth, second joint         |                    |                    |                      |                  |                                     |
| Replications                                                         | 2                  | 321.7**            | 0.04                 | 0.063*           | 6.50**                              |
| Treatments                                                           | 14                 | 39.0               | 0.10                 | 0.018            | 0.76                                |
| Error                                                                | 28                 | 37.7               | 0.17                 | 0.018            | 0.81                                |
| Total                                                                | 44                 |                    |                      |                  |                                     |
| Group C—Treatment May 27, 1948—Stage of growth, early heading        |                    |                    |                      |                  |                                     |
| Replications                                                         | 2                  | 154.7**            | 0.34**               | 0.018            | 2.56**                              |
| Treatments                                                           | 9                  | 16.7               | 0.23**               | 0.005            | 0.22                                |
| Error                                                                | 18                 | 14.2               | 0.05                 | 0.006            | 0.32                                |
| Total                                                                | 29                 |                    |                      |                  |                                     |
| Group D—Treatment June 23, 1948—Stage of growth, late dough          |                    |                    |                      |                  |                                     |
| Replications                                                         | 2                  | 148.8**            | 0.34**               | 0.013**          | 2.58**                              |
| Treatments                                                           | 9                  | 12.4               | 0.06                 | 0.002            | 0.26                                |
| Error                                                                | 18                 | 18.9               | 0.05                 | 0.001            | 0.41                                |
| Total                                                                | 29                 |                    |                      |                  |                                     |
| ANALYSES OF VARIANCE FOR ALL GROUPS                                  |                    |                    |                      |                  |                                     |
| Groups                                                               | 3                  | 418.0***           | 0.92**               | 0.079*           | 6.86                                |
| Treatments                                                           | 9                  | 27.7               | 0.18*                | 0.004            | 0.44                                |
| Replications within groups                                           | 8                  | 142.1***           | 0.26**               | 0.014*           | 2.48**                              |
| Groups $\times$ treatments                                           | 27                 | 16.3               | 0.09                 | 0.005            | 0.30                                |
| Error                                                                | 71                 | 25.0               | 0.08                 | 0.005            | 0.54                                |
| Total                                                                | 118                |                    |                      |                  |                                     |

\* Significant at the 5% level.

\*\* Significant at the 1% level.

\*\*\* Significant at the 0.1% level.

analyzed, the treatment differences for this group were no longer significant.

*Ash.* The statistical calculations (Table II) revealed no significant differences in the mineral content of the wheat as a result of 2,4-D applications. Differences due to replications, however, were significant within certain groups.

*Yield of Protein (Yield  $\times$  Protein).* Several workers (2, 7) have reported that 2,4-D treatments increased the protein content of wheat. For practical purposes, "yield of protein" representing both yield and protein content should also be considered, since it is recognized generally that the protein content of wheat is higher when grown under conditions causing reduced yields. It seems probable that the reported protein increase caused by 2,4-D applications was obtained at a sacrifice in yield. In the reports previously published (2, 7) protein content of 2,4-D treated plots have not been analyzed statistically nor discussed in relation to wheat yield data. However, in this work based on one year's study at one location, 2,4-D applications did not produce a significant difference in "yield" of protein.

*Milling and Baking Tests.* There was no indication that the milling quality of wheat was influenced by the spray treatments. Similarly the baking quality of the treated wheats was not impaired. This corroborates the work of Sibbitt and Harris (7). Samples in Group A (Table I) showed a slight increase in loaf volume with increasing concentrations of all three types of spray. Application of sprays at later stages of development did not produce similar effects, although the wheats produced were somewhat superior to those in Group A with respect to both protein content and loaf volume.

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# PREPARATION OF DRIED NATIVE WHEAT GLUTEN<sup>1</sup>

C. V. LUSENA

## ABSTRACT

A new method of preparing dried purified wheat gluten that retains its native properties consists of dispersing a washed gluten in 0.005 *N* acetic acid, centrifuging, and vac-ice drying the dispersion (lyophilizing). Washed gluten was used because the salts present in flour substantially reduced the solubility of gluten protein at concentrations of about 0.5 g. per liter of solution. Centrifuging removed the starch quantitatively. The dried gluten is a stable white powder containing 8% bound crude fat. Baking tests show that the purified gluten was substantially undamaged.

The preparation of suitable bulk samples of wheat gluten for research purposes is difficult and the usual practice has been to start each test with a separate portion of flour and either disperse it in a gluten solvent or use it to prepare a washed gluten for dispersion. This individual treatment of samples is not ideal because it may lead to considerable variation both in the purity of the gluten and in the extent to which it has been altered within a single series of tests.

The object of the present investigation was to prepare bulk samples of gluten for experimental purposes. Obviously, to be of value, the prepared gluten should be as near to its native state as possible, and it should be purified, stable, and easily handled. These requirements suggested that gluten should be dispersed in a mild solvent, purified, recovered from dispersion, dried, and, if necessary, ground.

Dilute acetic acid appeared to offer most promise as a gluten solvent. Neutral solvents have been considered milder (3) but the slow hydrolysis observed in acetic acid has been shown to be due to an enzyme (10). Concentrations of acetic acid as low as 0.01 *N* have been suggested (10). Strong acids at concentrations as low as 0.1 *N* have been used but they appear to be less satisfactory than weak acids (6). Recovery of gluten from dilute acetic acid dispersions demands only that the acetic acid be neutralized (11).

The literature is less informative on the best method of preparing gluten dispersions. The relation between lipoids and gluten is complex; ether will extract fats from normal flour but not from well moistened flour (9). Dispersions have been prepared in dilute acetic acid directly from flour (6), but other investigators have been unable

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to use flour directly (11). Salts, even at low concentrations, affect the solubility of gluten in acids (14). The more common practice is to prepare crude gluten by making flour into a dough and then washing it in tap water or buffer solution. The resulting mass requires either a long contact time with the solvent or mechanical treatment. The use of the latter has been criticized on the basis that it changed the shape of the ultracentrifuge sedimentation diagrams (7), but as the treatment given involved precipitation and redispersion, the evidence against mechanical treatment is not conclusive. In view of the apparent contradictions in the literature, preparations of dispersions from flour and from washed gluten were compared.

Gluten dispersions and in some cases recovered gluten were dried by freezing the mixture and removing the moisture under vacuum. This method, sometimes called lyophilization, is referred to in this paper as vac-ice drying.

The final step was to determine if the purified gluten had retained its native characteristics. Probably the most comprehensive test of gluten quality is the baking test; it has been used to evaluate the quality of crude gluten (1, 4, 5). Consequently, in this study, the gluten preparations were subjected to baking tests. A recommended procedure of preparing purified gluten is given at the end of this paper.

### Materials and Methods

The flour was a commercial first patent flour containing 12.8% protein (14% moisture basis), prepared from Western Canadian Spring Wheat No. 1 and No. 2 Northern. The manufacturer revealed that it had been aged with nitrogen trichloride (Agene) and bleached with benzoyl peroxide (Novadelox). As most investigators have used unmodified (non-extracted) flour the fats were not extracted from the flour in this study.

Nitrogen contents were determined by micro or macro Kjeldahl methods. Moisture content was determined by drying to constant weight at 75°C. at less than 1 mm. of mercury pressure, ash by ignition at 600°C., and starch polarimetrically (2). Crude fat was determined as follows: the samples were extracted with absolute ethanol for 18 hr. in a Soxhlet extractor, the extract was evaporated to dryness at 100°C., the residue was extracted with chloroform, the chloroform extract was filtered and dried at 65°C. overnight and weighed as crude fat. Hydrogen ion concentration was determined electrometrically using a glass electrode. Concentrated gluten dispersions were diluted to approximately 1 mg. of nitrogen per ml. of dispersion prior to determining the pH; otherwise the protein coagulated on the electrodes. Diluting dispersions containing 10 mg.

of nitrogen per ml. had no effect on the pH value obtained, so it was assumed that diluting more concentrated dispersions did not alter the pH appreciably.

The baking formulas employed by Aitken & Geddes (1) were used.

## Results

*Choice of Material for Dispersion.* Preliminary tests showed that crude gluten would disperse almost quantitatively in 0.01 *N* acetic acid to give dispersions containing 8 mg. of nitrogen per ml. of dispersion (4.5% gluten) but when flour containing the amount of gluten needed to give a dispersion of this concentration was used, only a small fraction of the gluten protein dispersed. The cause of this was traced to a water-soluble material since making flour into a dough did not increase the amount of protein which dispersed in acetic acid, whereas stirring flour in water, centrifuging, and discarding the liquid increased the amount of gluten that dispersed in acetic acid to the same value as when crude gluten was used.

The effect of water-soluble flour components on the solubility of gluten in 0.01 *N* acetic acid was investigated further. The material precipitated by 70% alcohol from an aqueous extract of flour had no effect on the solubility of gluten in 0.01 *N* acetic acid, nor did the material left after dialysing an aqueous extract of flour. Various 0.01 *N* acetic acid solutions containing different amounts of ash were prepared by adding aqueous extract of flour, the 70% alcohol soluble fraction of an aqueous extract, the dialysate of an aqueous extract, the ash from an aqueous extract of flour, and salts to simulate a synthetic ash.<sup>2</sup> Then 5.0 g. portions of vac-ice dried gluten (prepared by a procedure given later) were dispersed in 100 ml. portions of the various solutions with a Waring Blendor. The vac-ice dried gluten dispersed in aqueous acetic acid in less than 2 min. and was not affected by prolonged stirring; a 5 min. mixing time was allowed with the acetic acid solutions containing ash so that a slower rate of dispersion would not be confused with insolubility. The results in Fig. 1 show that, irrespective of the source of ash, a concentration of 0.5 to 0.75 g. per liter was sufficient to reduce substantially the solubility of gluten. The pH of the dispersions did not vary enough to have any effect on the amount of gluten dispersed.

These results show the necessity of removing the soluble ash from flour prior to dispersing the gluten. Starch does not decrease the solubility of gluten but it increases the amount of the dispersion which is lost during purification. Consequently, crude gluten was selected as the best starting material for gluten dispersions.

<sup>2</sup> One liter of salt solution contained 1.67 g. of  $\text{KH}_2\text{PO}_4$ , 0.47 g. of  $\text{Mg}_3(\text{PO}_4)_2 \cdot 4\text{H}_2\text{O}$ , 0.31 g. of  $\text{Na}_2\text{SO}_4$ , 0.23 g. of  $\text{MgSO}_4$ , 0.17 g. of  $\text{CaSO}_4$ , 0.12 g. of  $\text{MgCl}_2$  and 0.03 g. of  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ .



*Dispersion of Crude Gluten.* Crude gluten was dispersed in 0.01 and 0.005 *N* acetic acid with a Waring Blendor and at the end of the mixing time five drops of *n*-octyl alcohol were stirred in. Since the toughness of crude gluten is increased by a more complete removal of the starch and since the extent of mechanical treatment required is obviously related to the tenacity of the crude gluten, tests were undertaken to establish the best washing conditions. Three samples of wet gluten were prepared that contained, on analysis, 42%, 25%, and 6% starch respectively, on a dry basis. Portions of each, sufficient to

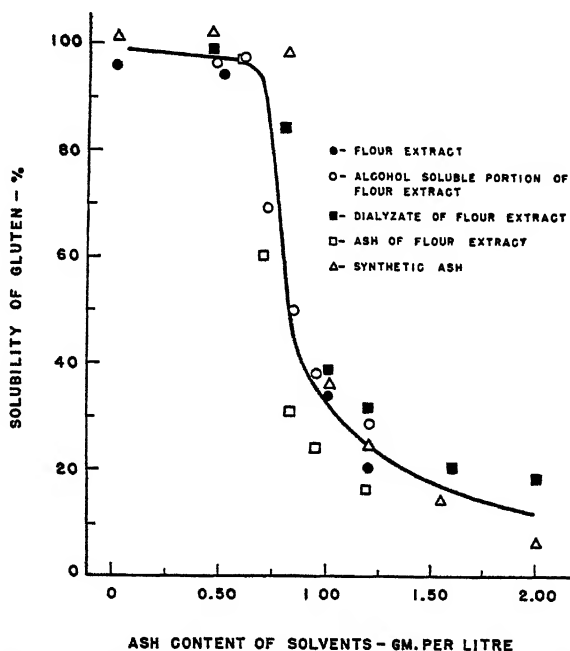


Fig. 1. Effect of salts on the solubility of gluten in 0.01 *N* acetic acid.

give approximately 4% gluten dispersions, were mixed with the solvents in a Waring Blendor for varying periods and the proportion dispersed was determined. The results, recorded in Table I, show that except for the toughest gluten (Sample 3), 3 min. stirring was sufficient. Large particles of Sample 3 were apparent at the end of this period, but after 7 min. stirring it gave the highest percentage dispersion. Tests showed that the lower percentage dispersion with the other samples was not due to salts, and therefore, the more complete dispersion of Sample 3 is attributed to a more thorough removal of acid-insoluble components during washing. Sample 1 also gave a higher loss of gluten during purification owing to the amount adhering to the starch, which was removed by centrifuging.

The gluten containing 25% starch was considered satisfactory and a stirring time of 5 min. was ample. The gluten washing procedure developed by Shewfelt and Adams (14) yields a gluten containing approximately 25% starch and was accordingly adopted. Details are given in the last section of this paper.

As 0.005 *N* acetic acid disperses gluten, it seemed worthwhile to determine if dispersion were a function of pH alone. Dispersions were prepared in 0.0025, 0.005, and 0.01 *N* hydrochloric acid. The first failed to disperse the same amount as 0.005 *N* acetic acid. The pH of a dispersion in 0.005 *N* hydrochloric acid containing 7.0 mg. of nitrogen per ml. of dispersion was 4.9 as compared to 5.1 in 0.005 *N* acetic acid. This similarity suggested that pH was the important factor.

*Purification of Gluten Dispersions.* Preliminary tests on dispersions containing 7 mg. nitrogen per ml. of dispersion showed that centrifug-

TABLE I  
EFFECT OF STARCH CONTENT ON THE DISPERSABILITY  
OF GLUTEN IN ACETIC ACID

| Sample | Starch content, % dry matter | Protein dispersed in 0.005 <i>N</i> acetic acid, % |                       | Protein dispersed in 0.01 <i>N</i> acetic acid, % |                       |                       |
|--------|------------------------------|----------------------------------------------------|-----------------------|---------------------------------------------------|-----------------------|-----------------------|
|        |                              | After 3 min. stirring                              | After 5 min. stirring | After 3 min. stirring                             | After 5 min. stirring | After 7 min. stirring |
| 1      | 42                           | 94.4                                               | 94.6                  | 95.0                                              | 95.1                  | 95.0                  |
| 2      | 25                           | 96.2                                               | 96.3                  | 96.3                                              | 96.0                  | 96.2                  |
| 3      | 6                            | 10                                                 | 93.3                  | 15                                                | 93.6                  | 98.1                  |

ing in bottles of 200 ml. capacity at 2,000 times gravity for 10 min. effectively sedimented undispersed material. As purification was probably related to viscosity, i.e., to gluten concentration, different dispersions were centrifuged at the same rate and for the same time and their starch contents determined. Cooling the dispersions during preparation increased the starch content of the purified gluten, therefore, extracts were made and centrifuged at room temperature. The results recorded in Table II show that the starch content, expressed as a percentage of the dry matter, remained almost constant up to a concentration of 7 mg. nitrogen per ml. but increased with concentration thereafter. Longer centrifuging would have purified more concentrated dispersions, but for convenience, 7 mg. nitrogen per ml. of dispersion was accepted as a suitable concentration. Purification by precipitation and dispersion is reported later.

*Recovery of Gluten.* Gluten is readily salted out from an acetic acid dispersion, but as this would contaminate the gluten with salt, it

TABLE II  
EFFECT OF GLUTEN CONCENTRATION ON PURIFICATION

| Concentration of acetic acid, <i>N</i> | Concentration of dispersion, mg. nitrogen per ml. | pH of dispersion | Starch content of gluten, %, dry matter basis |
|----------------------------------------|---------------------------------------------------|------------------|-----------------------------------------------|
| 0.005                                  | 4.2                                               | 4.7              | 0.06                                          |
|                                        | 5.5                                               | 4.9              | 0.06                                          |
|                                        | 6.1                                               | 5.0              | 0.06                                          |
|                                        | 6.7                                               | 5.0              | 0.06                                          |
|                                        | 7.9                                               | 5.2              | 0.10                                          |
| 0.01                                   | 4.3                                               | 4.5              | 0.06                                          |
|                                        | 5.6                                               | 4.6              | 0.06                                          |
|                                        | 6.0                                               | 4.7              | 0.05                                          |
|                                        | 6.7                                               | 4.8              | 0.06                                          |
|                                        | 7.4                                               | 4.8              | 0.07                                          |
|                                        | 8.1                                               | 4.8              | 0.09                                          |
|                                        | 8.8                                               | 4.9              | 0.09                                          |
|                                        | 9.3                                               | 4.9              | 0.08                                          |

was abandoned in favor of neutralizing the acetic acid with saturated calcium hydroxide solutions. When gluten dispersion was added to calcium hydroxide solution the pH of the supernatant liquid was lower than when the alkali was added to the dispersion. Above pH 9.5, gluten redispersed. To avoid high local concentrations, either a Waring Blendor or an ordinary stirrer was employed.

The relation between pH, as influenced by the addition of calcium hydroxide solution, and gluten recovery with each stirring method is shown in Fig. 2. At the lower pH values, recovery was less with the mechanical stirrer—probably because the protein had a lower pH than the liquid—but at pH 6.5, and higher, recovery was better with the stirrer; the Waring Blendor tended to redisperse the gluten as alkalinity increased. The mechanical stirrer also had the advantage

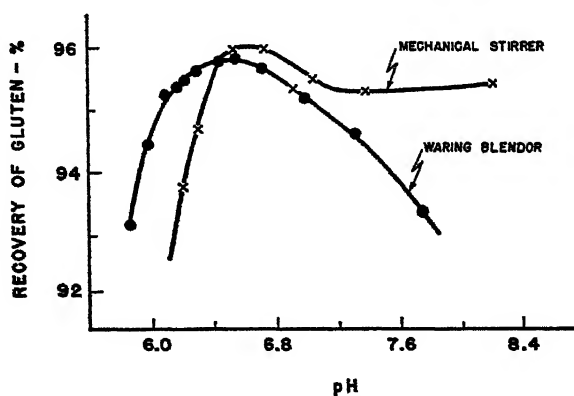


Fig. 2. Effect of pH on the recovery of gluten.

that all but about 0.5% of the gluten collected on the stirrer shaft and was easily removed. (The remaining 0.5% can be centrifuged out and collected if desired.) The stirrer is therefore preferred during precipitation. Pressing and draining the gluten made it more tenacious and greatly interfered with redispersion.

*Second Dispersion and Purification.* With a Waring Blendor, a number of dispersions of soft (unpressed) gluten in 0.01 *N* acetic acid were prepared from one sample of recovered gluten. The amounts used varied from 50 to 250 g. of wet gluten per 250 ml. of 0.1 *N* acetic acid. Over 99.5% of all the amounts up to and including 200 g. dispersed. As the gluten contained water, 200 g. would dilute the acetic acid to about 0.006 *N*. The dispersions were centrifuged and the amount of sedimented starch determined. The results given in Table III show that the amount of starch sedimented decreased as the

TABLE III  
DISPERSION AND PURIFICATION OF RECOVERED GLUTEN

| Conc. of dispersion, mg.<br>nitrogen per ml. | pH of dispersion | Gluten dispersed, % | Starch sedimented,<br>% by weight of gluten |
|----------------------------------------------|------------------|---------------------|---------------------------------------------|
| 8.5                                          | 5.1              | 99.7                | 0.12                                        |
| 10.3                                         | 5.2              | 99.8                | 0.11                                        |
| 11.5                                         | 5.3              | 99.6                | 0.11                                        |
| 12.7                                         | 5.3              | 99.7                | 0.08                                        |
| 14.5                                         | 5.4              | 99.7                | 0.07                                        |
| 16.1                                         | 5.5              | 99.6                | 0.05                                        |
| 17.1                                         | 5.5              | 99.2                | 0.04                                        |
| 19.1                                         | 5.5              | 99.1                | 0.04                                        |
| 21.0                                         | 5.6              | 99.7                | 0.04                                        |
| 23.4                                         | 5.6              | 99.6                | 0.04                                        |
| 19.9                                         | 5.7              | 65.0                | —                                           |

gluten concentration increased. A concentration of 10–12 mg. nitrogen per ml. of dispersion permitted satisfactory purification and further dilution had no effect on the amount of starch which was sedimented. The results also show that up to 23 mg. of gluten nitrogen per ml. can be dispersed. No gluten sedimented out during 10 days cold storage.

Repeated recovery and dispersion of gluten appeared to remove a non-gluten nitrogenous substance; after the first recovery 4–5% of the nitrogen remained in the supernatant liquid, but with repeated recovery and dispersion this value fell to about 1% and the concentration of nitrogen in the supernatant liquid dropped to, and remained at, 0.12–0.14 mg. nitrogen per ml. This probably represents the solubility of gluten in the supernatant liquid.

*Vac-ice Drying of Gluten.* Dispersions containing 8–23 mg. nitrogen per ml. were poured into aluminum trays to a depth of 0.5 cm. and

frozen at  $-18^{\circ}\text{C}$ . This required about 2 hr. The trays were placed in a vacuum chamber on heavy metal shelves and the pressure maintained below 1 mm. of mercury. To counteract cooling due to evaporation, heat was applied electrically to maintain the temperature of the shelves at  $-1^{\circ}\text{C}$ . The evaporated moisture was condensed on a coil which was held at  $-20^{\circ}\text{C}$ . Drying required 36 to 48 hr. The gluten remained as a white layer which showed the crystal pattern of ice. It was readily crumbled into a powder when passed through a 40-mesh sieve.

*Properties of Vac-ice Dried Gluten.* The vac-ice dried gluten readily formed a tenacious glutenous mass when mixed with water in the ratio of 1 g. of gluten to 1.5 ml. water. It dispersed quantitatively in distilled water up to concentrations of 17 mg. nitrogen per ml. (with a Waring Blendor) and the pH of the dispersions was below 5.0. This solubility and the low pH were attributed to bound acetic acid. Acetic acid evaporates readily under the conditions of vac-ice drying in the absence of gluten, but analyses showed that gluten dried from dispersions retained about 0.5% acetic acid. On neutralizing the gluten, by adding dilute sodium hydroxide, the solubility decreased to 0.14 mg. nitrogen per ml., i.e., to the solubility of recovered gluten in distilled water. The pH of minimum solubility was 6.55, which is the same as that of recovered gluten.

A typical analysis of a preparation follows:—

|                                  | <i>Dry matter basis, %</i> |
|----------------------------------|----------------------------|
| Protein (nitrogen $\times 5.7$ ) | 90.0                       |
| Crude fat (alcohol soluble)      | 8.0                        |
| Ash                              | 0.5 (or less)              |
| Acetic acid                      | 0.5                        |
| Starch                           | 0.01 (or less)             |

Dry matter was normally above 92%. The crude fat in the gluten was not ether-soluble.

*Baking Quality Tests.* To determine the effect of varying the conditions of preparation of gluten, 12 samples were made. The pH of the first dispersion was varied from 4.8 to 5.2 by changing the concentration of acetic acid and the pH of the second dispersion, from 4.3 to 5.5. One sample was recovered and vac-ice dried, the others were vac-ice dried directly from dispersion.

A soft white spring wheat flour containing 8.0% protein (14% moisture basis) was used as the base flour for baking tests and gluten preparations were added to increase the protein to 13.5%. As a control, an air-dried crude gluten was prepared from the same flour by the method of Aitken and Geddes (1) as they have shown that this method gives an undamaged gluten. Preliminary trials showed that the malt-phosphate formula was more satisfactory than the malt-

phosphate-bromate formula as it gave loaf volumes 40 cc. higher with the base flour, 45 cc. higher with the base flour plus control gluten, and 80 cc. higher with the base flour plus purified gluten. Examination of the physical appearance of the crust showed that the loaves had a characteristic "over-aged" appearance. The possibility was tested that *n*-octyl alcohol (which was used as an antifoam) might interfere but the amount that could be present (even if none were lost during drying) had little effect on the volumes of loaves made from an ordinary flour.

TABLE IV  
EFFECT ON BAKING QUALITY OF INCREASING THE PROTEIN CONTENT OF A  
SOFT WHEAT FLOUR TO 13.5% WITH VARIOUS GLUTEN PREPARATIONS

| Gluten preparation | Description of added gluten                | pH, first dispersion | pH, second dispersion | Loaf volume, cc. | Crumb |         |
|--------------------|--------------------------------------------|----------------------|-----------------------|------------------|-------|---------|
|                    |                                            |                      |                       |                  | Color | Texture |
|                    | None added                                 | —                    | —                     | 450              | 4.5   | 4.5     |
|                    | Control gluten (air dried)                 | —                    | —                     | 680              | 4.5   | 4.5     |
| 1                  | Recovered and dried                        | 4.8                  | —                     | 655              | 5.0   | 6.0     |
| 2                  | Dispersion dried                           | 4.8                  | 5.5                   | 660              | 5.0   | 5.5     |
| 3                  | As 2, neutralized                          | 4.8                  | 5.5                   | 665              |       |         |
| 4                  | Dispersion dried (Stored 6 months at 5°C.) | 5.0                  | 5.2                   | 695              | 6.0   | 6.0     |
| 5                  | Dispersion dried                           | 5.2                  | 5.5                   | 705              | 5.0   | 6.0     |
| 6                  | Dispersion dried                           | 4.8                  | 5.6                   | 640              | 5.0   | 5.5     |
| 7                  | Dispersion dried                           | 4.8                  | 5.2                   | 620              | 5.0   | 5.5     |
| 8                  | Dispersion dried                           | 4.8                  | 5.1                   | 630              | 5.0   | 5.5     |
| 9                  | Dispersion dried                           | 4.8                  | 4.9                   | 615              | 5.0   | 5.5     |
| 10                 | Dispersion dried                           | 4.8                  | 4.7                   | 600              | 4.5   | 5.5     |
| 11                 | Dispersion dried                           | 4.8                  | 4.5                   | 565              | 4.0   | 5.5     |
| 12                 | Dispersion dried                           | 4.8                  | 4.3                   | 540              | 4.0   | 5.5     |

Preparations 1-12 were vac-ice dried.

All 1st dispersions were in 0.01 *N* acetic acid with the exception of Nos. 4 and 5 where 0.005 *N* acetic acid was used.

The 2nd dispersion for No. 2 to No. 6 were in 0.01 *N* acetic acid. For the subsequent samples the acid concentration was proportionately increased to 0.05 *N*.

Baking results are means of duplicates.

As the malt-phosphate formula gave higher loaf volumes it was used in the main series. Duplicate loaves were baked and the mean results are recorded in Table IV. The baking results suggest that the best gluten preparations, Nos. 4 and 5, were slightly better than the control gluten. Dried recovered gluten, No. 1, was not significantly different from dried dispersed gluten, No. 2. Neutralized gluten, No. 3, was little better than No. 2. Preparation No. 4, which had been stored for six months at 5°C., was similar to a new sample, No. 5. Increasing acid concentration both in 1st and 2nd dispersions affected the baking qualities of the gluten adversely. For comparison, a hard red spring wheat flour containing 13.5% protein was baked; the loaf volumes were 715 cc. by the malt-phosphate formula and 815 cc. by the malt-phosphate-bromate formula.

### Discussion

The effect of salts on the dispersion of gluten in dilute acetic acid has been shown. The critical concentration of salts which interferes with the dispersion of gluten is about 0.5 g. per liter. If a flour containing 0.5 ash and 10% gluten is used directly, the soluble salts present will interfere with the preparation of dispersions containing more than 1.4% gluten, i.e., 2.5 mg. of nitrogen per ml. of dispersion. Below this concentration normal flour does not contain enough soluble salts to interfere. This probably accounts for some of the inconsistencies in the literature. It is significant that those who have used flour directly have prepared only dilute gluten dispersions.

In the preparation of purified gluten of good baking quality the pH of the dispersion is important. That of the first dispersion should be 5.0 or higher and that of the second 5.5 or higher. Neither the dispersion of gluten nor its recovery reached 100%; the former is attributed not to the presence of salts but to an acid-insoluble material that is removed on washing, and the latter to a water soluble non-gluten fraction that has been recognized by others (8, 13) and also to the slight solubility of gluten.

The loaf volume for a "strong" flour by the malt-phosphate formula was only 10 cc. higher than that of a flour fortified with the best gluten (No. 5, Table IV). The addition of bromate increases the loaf volume of a strong flour by 100 cc., but it decreased the loaf volume of a vac-ice gluten fortified flour by 80 cc. and that of a control gluten fortified flour by 45 cc. These results and the "over-aged" appearance of the crust are partly due to "aged" flour being used to prepare the gluten. Some difference in bromate response between the "fortified" flour and that of a "strong" wheat flour would be expected because part of the protein of the former is of poorer "quality" than that of the latter. Since the control gluten was not affected adversely to the same extent as the purified gluten, this would suggest that the purified gluten was slightly modified. Further studies on gluten from laboratory milled flours and on fat-free flours are needed.

### Recommended Procedure for Preparing Purified Dried Gluten

1. *Gluten Preparation.* Mix 1,000 g. flour and 800 ml. tap water at 30°C. for 20 min. in a suitable dough mixer. Add 3,000 ml. tap water and stir for 2 min. with a high speed stirrer. Separate the gluten on a screen and drain to approximately 500 g. Divide into eight portions.

2. *Dispersion.* Disperse each portion in 250 ml. of 0.005 *N* acetic acid for 5 min. with a Waring Blendor. Stir in five drops *n*-octyl

alcohol at the end of mixing. Check the pH (5.0–5.2). (Total volume 2,500 ml., concentration about 8 mg. of nitrogen per ml. of dispersion.)

3. *Purification.* Centrifuge for 10 min. at 2,000 times gravity. (A continuous centrifuge can be used but conditions must be established that reduce the starch to the desired level.)

4. *Recovery.* Stir the combined dispersions and add 14 ml. saturated (filtered) calcium hydroxide per 100 ml. dispersion. Check the pH of the supernatant liquid and titrate to pH 6.5–6.8. Recover the gluten from the stirrer and by screening, if necessary. Further handling interferes with the following steps. Divide into four portions.

5. *Second Dispersion and Purification.* Disperse each portion in 250 ml. 0.01 *N* acetic acid for 5 min. with a Waring Blendor. Stir in 5 drops *n*-octyl alcohol at the end of mixing. (pH 5.5–5.7, total volume 1,500 ml., concentration about 13 mg. nitrogen per ml. dispersion.) Centrifuge and recover as before. N.B.—If freedom from starch is not important, the concentration can be increased to 20 mg. per ml. and this reduces the drying load.

6. *Vac-ice Drying.* Pour the dispersion into rust-resistant metal trays to a depth of 0.5 cm., freeze, and while it is still frozen evaporate at low pressure. Heat has to be applied to counteract cooling due to evaporation and the temperature should be maintained at  $-1^{\circ}\text{C}$ . Drying requires 24–36 hr. if the pressure is kept below 1 mm. of mercury. The product can be rubbed through a 40-mesh sieve.

7. *Storing.* As a precaution, store the gluten in sealed containers in a refrigerator at about  $5^{\circ}\text{C}$ .

N.B.—Steps 1–5 inclusive do not require more than 3 hr.

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## NUMBERS, KINDS, AND SOURCE OF MOLDS IN FLOUR<sup>1</sup>

CLYDE M. CHRISTENSEN and MORTIMER COHEN<sup>2</sup>

### ABSTRACT

The mold counts of approximately five hundred samples of flour, collected principally in commercial mills, ranged from several hundred to more than 5,000 per g. Three samples of washed wheat from one mill, collected as the wheat went to the first break rolls, contained only a few hundred molds per g., while the flours milled from these wheats contained up to several thousand molds per g. Flour collected from the interior of spouts, roll housings and other mill machinery bore from several thousand to several million molds per g., of the same species as were commonly encountered in commercial flours. The chief source of mold contamination of flour appears to be molds growing and sporulating within the milling system itself. The predominant molds in most of the flours were *Aspergillus glaucus* and *A. candidus*. Unidentified species of *Penicillium* made up a major portion of the mold flora in only a few samples of commercial flour. Several other genera were found in most samples, but only in small numbers.

The evidence now available, if not extensive, is at least sufficient to indicate that freshly milled flour bears considerable numbers of different bacteria, yeasts, and molds. The literature dealing specifically

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with molds in flour is scanty. Barton-Wright (1) stated that freshly milled flours contained on the average from 1,000 to 2,000 molds per g. The medium he used was not designed specifically to select molds, and so the counts he reported probably were somewhat low. Christensen (2) compared several technics and a number of different media for the purpose of determining mold counts of flour. He found malt-salt agar to be preferable to the others tested, primarily because it resulted in larger counts and a greater number of species, in the majority of samples, than any of the other media tested. James and Smith (4) in a rather thorough study of the microflora of five Canadian flours, obtained a higher mold count on malt-salt agar than on Czapek's agar in all five samples. They emphasized the need for adequate replicates, as well as suitable sampling technics and selective media, in determining the microfloral population of flour. The chief aims of the present work were to determine the number and kinds of molds present in commercial wheat flours, and to explore some of the possible sources of this contamination. The data were obtained from assays of approximately five hundred samples of flour, from 16 mills, over a period of nearly four years, and thus is to be considered only as a moderately extensive preliminary survey.

### Materials and Methods

*Collection of Samples.* Most of the samples of flour were collected at the mills, although a few were obtained from bakeries. At the mills, the flour from different streams usually was obtained by inserting a small, sterile paper bag into the spout to get the amount wanted. With a few exceptions, each sample comprised from 4 to 16 ozs.

*Determining Mold Population.* One gram of flour was weighed on sterile filter paper on a torsion balance sensitive to 0.01 g., placed in a 4 oz. bottle containing 10 g. of sterile quartz sand and 100 ml. of sterile water to which a wetting agent had been added in concentration of approximately 1:10,000. Previous tests had indicated that the wetting agent in this concentration had no inhibitory effect on germination or growth of the molds concerned. The mixture was then shaken 200 times, and 1 ml. portions immediately pipetted into each of two to four sterile petri dishes. Twenty ml. of malt-salt agar (20 g. malt extract, 20 g. agar, 75 g. NaCl, 1 liter of water) cooled to 48-50°C. were added, the dishes swirled to distribute the flour through the agar, and allowed to stand until the agar hardened. The dishes were then stacked and covered to exclude air contaminants. Occasionally flour sterilized by heating to 180°C. for 4 hours was similarly sampled as a control; these seldom averaged one mold colony per plate, indicating that chance contamination was negligible.

The dishes were incubated in a laboratory at temperatures of 21–23°C. Colonies were counted after five to seven days. In most cases the dishes were examined both with the naked eye and microscopically, at magnifications of 10× to 30×.

*Variation in Mold Counts of Flour from Different Mills.* The mold counts of flours from 16 mills in 15 cities of 9 different states are presented in Table 1. A number of different types of flour were included, so that data probably indicate only the general range of mold contamination that is to be encountered. Some of these samples were

TABLE 1  
MOLD COUNTS OF WHEAT FLOURS FROM DIFFERENT MILLS

| Mill No | Location  | Type of flour | Date collected     | Molds per g |
|---------|-----------|---------------|--------------------|-------------|
| 1       | Idaho     | Cake          | Aug. '46           | 200         |
| 2       | Idaho     | Bakers Patent | Aug. '46           | 500         |
| 3       | Utah      | Bakers Patent | Aug. '46           | 300         |
| 4       | Colorado  | Bakers Patent | Aug. '46           | 800         |
| 5       | Colorado  | Family Patent | Aug. '46           | 1,400       |
| 6       | Kansas    | Bakers Patent | Aug. '46           | 800         |
| 7       | Kansas    | Family Patent | Aug. '46           | 1,400       |
| 8       | Kansas    | Bakers Patent | Aug. '46           | 4,000       |
| 9       | Missouri  | Patent        | Aug. '46, sample 1 | 2,200       |
|         |           |               | sample 2           | 3,100       |
| 10      | Missouri  | Sponge        | Aug. '46           | 2,900       |
| 11      | Missouri  | Bakers Patent | Aug. '46, sample 1 | 2,600       |
|         |           |               | sample 2           | 3,300       |
|         |           |               | sample 3           | 4,800       |
|         |           |               | sample 4           | 5,200       |
| 12      | Minnesota | Clear         | Aug. '45           | 4,700       |
|         |           |               | Nov. '45           | 1,100       |
|         |           |               | Nov. '45           | 900         |
| 13      | Minnesota | Family Patent | July '45           | 200         |
| 14      | S. Dakota | Bakers Patent | Nov. '46           | 600         |
|         |           |               | Dec. '47           | 1,800       |
|         |           |               | Jan. '48           | 3,200       |
|         |           |               | Jan. '49           | 400         |
| 15      | Oregon    | Bakers Patent | Jan. '49           | 1,500       |
| 16      | New York  | Bakers Patent | Aug. '46, sample 1 | 900         |
|         |           |               | sample 2           | 800         |

obtained from mills, some from bakeries. In all cases the moisture content of the flour was considered to be too low to permit significant increase in molds during the time that elapsed between samples and assaying them.

*Variation in Mold Counts among Representative Streams.* Fourteen streams in each of two mills and 21 in another were assayed, the first two in November, 1945, and the third in September, 1946. The data in Table 2 were selected from these samplings to illustrate the general range of mold contamination in the various streams of each of the three mills.

TABLE 2  
MOLD COUNTS OF FLOUR FROM REPRESENTATIVE STREAMS IN THREE MILLS

| Streams       | Mill   |       |       |
|---------------|--------|-------|-------|
|               | 1      | 2     | 3     |
| 1st break     | 66,000 | 2,500 | 2,800 |
| 2nd break     | 8,400  |       | 1,200 |
| 4th break     | 3,800  | 6,600 | 800   |
| 1st middlings |        |       | 300   |
| 2nd middlings |        |       | 500   |
| 6th middlings |        |       | 200   |
| 1st scalp     |        | 3,900 |       |
| 3rd scalp     |        | 6,400 |       |
| 1st tailings  | 6,900  |       | 1,000 |
| 2nd tailings  | 7,400  | 9,200 | 1,700 |

*Variation in Mold Counts among Different Streams of the Same Mills, and within the Same Stream at Different Times.* In one mill, all of the streams that contributed to the "first clear" were sampled at intervals of four to eight hours through a period of 24 hours, during January, 1947, and the bran and shorts streams were sampled at the beginning and end of the 24 hour period. The aim was to determine whether some streams were contributing significantly more molds than others to the final product, and also to determine whether the portion of the seed going into flour had a higher or lower mold count than the portions not going into flour. Approximately 80 samples were assayed, and typical data are presented in Table 3. These were chosen to indicate the degree of mold contamination encountered, in this single test, in the various streams that contributed to the final flour and those that made up the portions not going into flour.

*Variation within a Given Sample.* To determine the variation within a given sample, five additional one-gram portions of the first middlings flour collected at 3 P.M. in January, 1947 (included in Table 3) were assayed. According to the first assay, this flour had a

TABLE 3  
MOLD COUNTS OF THE SAME STREAMS IN ONE MILL AT INTERVALS  
OF 4 TO 24 HOURS, JANUARY 21-22, 1947

|                  | 1st Bk.<br>flour | 1st mid-<br>dlings flour | 1st tailings<br>flour | 1st clear<br>flour | Bran  | Shorts |
|------------------|------------------|--------------------------|-----------------------|--------------------|-------|--------|
| Jan. 21, 11 A.M. | 6,800            | 3,400                    | 4,700                 | 4,600              | 1,200 | 3,200  |
| 3 P.M.           | 4,300            | 800                      | 6,400                 |                    |       |        |
| 7 P.M.           | 5,700            | 1,700                    | 6,300                 |                    | 2,600 |        |
| 11 P.M.          | 8,900            | 2,400                    | 4,600                 |                    |       |        |
| Jan. 22, 3 A.M.  | 12,800           | 5,000                    | 8,300                 |                    | 3,000 |        |
| 11 A.M.          | 8,000            | 1,400                    | 6,200                 | 3,300              | 700   | 6,200  |

count of 800 molds per g. Five plates were used for each of the five replicates. As determined by these replicates, the mold count of this sample was, respectively, 800, 1,000, 1,050, 1,100, and 1,350 per g., with an average of 1,050. Another sample which, according to the original assay, had a mold count of 7,200 per g., was assayed by five different workers. Their results on this sample were 7,000, 6,800, 7,000, 7,300 and 7,200 per g.

Thus, variations due to technic and to unequal distribution of spores within the sample probably may be expected to fall within a range of 5% to 20%. If dilutions are used that result in 20 to 50 colonies per plate, as was the case in most of the present work, the variations between replicates of the same sample usually amounts to no more than plus or minus 5% to 10%.

*Source of the Molds Present in Flour.* Evidence presented elsewhere (2) shows that wheats, as they come to the mill, may have a mold

TABLE 4  
MOLD COUNTS OF 3 SAMPLES OF WHEAT BEFORE AND AFTER WASHING,  
AND OF TYPICAL PRODUCTS MILLED FROM THEM

|                      | Wheat sample number |       |       |
|----------------------|---------------------|-------|-------|
|                      | 1                   | 2     | 3     |
| Wheat before washing | 2,800               | 3,100 | 1,900 |
| Wheat after washing  | 300                 | 400   | 500   |
| Tempered wheat       | 300                 | 400   | 500   |
| Bran                 | 2,600               | 1,900 | 1,200 |
| Shorts               | 3,200               | 4,700 | 2,700 |
| Patent               | 1,800               | 3,200 | 400   |

count of from 2,000 to 5,000 or more per g. Data gathered in the present tests support the contention that this is a "normal" mold contamination of high grade wheats as they arrive at the mill. To determine whether these molds on the wheat seed were contributing significantly to the mold contamination of flour milled from the same seed, the mold counts of unwashed, washed, and tempered wheats, and of the various fractions milled from these wheats, was determined. The results are presented in Table 4.

While the data are limited in number, they are from samples gathered at approximately annual intervals and thus represent different varieties of wheat grown under widely different environmental conditions in the Great Plains area. Evidently, the mold count of high quality wheat, after washing, usually amounts to only a few hundred per g. The greater mold counts of the bran and shorts as compared with that of the washed wheat might be explained by the

hypothesis that most of the mold present in the seed is present as mycelium in the outer layers of the pericarp. In at least one of the samples, however, 100% of the mold count of the washed wheat was *Alternaria*, but *Alternaria* did not make up more than 5% of the count of the bran or shorts milled from this wheat. Also, the relatively high count of molds in two of the samples of patent flour obtained from wheat with a low mold flora as it went to the break rolls could not be explained by such a hypothesis. This suggested that molds common in flour were increasing and multiplying within the milling system itself.

To test this, various samples of flour were collected from the interior of roll housings, spouts, conveyers, and other places where the

TABLE 5  
MOLD COUNTS OF FLOUR ADHERING TO INTERIOR OF MILL MACHINERY

| Mill | Source of flour                                               | Molds per g |
|------|---------------------------------------------------------------|-------------|
| 1    | Conveyer of 8½ reel                                           | 88,000      |
| 2    | Break roll housing                                            | 1,117,000   |
| 3    | First break reel                                              | 16,000      |
|      | Inner side of cover of inspection opening in 2nd break shaker | 1,500,000   |
| 4    | 1st break roll housing                                        | 4,700       |
|      | 2nd break roll housing                                        | 11,000      |
|      | 3rd break roll housing                                        | 726,000     |
|      | 4th break roll housing                                        | 13,000      |
|      | 5th break roll housing                                        | 2,456,000   |
|      | 2nd mids. red. roll housing                                   | 364,000     |
|      | 3rd mids. red. roll housing                                   | 892,000     |
|      | 4th mids. red. roll housing                                   | 5,000       |
|      | 5th mids. red. roll housing                                   | 1,014,000   |
|      | 6th mids. red. roll housing                                   | 1,020,000   |
|      | Sizings reduction roll housing                                | 3,360,000   |

relative humidity might be high enough to favor the increase of molds. The results are presented in Table 5.

The molds cultured in large numbers from this material were of the same species as those encountered as major contaminants in commercial flours from these and other mills. Most of the mold contamination of commercial flours that we have sampled appears to come from within the mill machinery itself. Additional proof of this is offered by a summary of the major types of mold contaminants of the flours we have assayed, as given below.

*Principal Kinds of Molds Cultured from Commercial Flours.* Approximately 20 species of molds, in eight genera, have been found in the flours so far sampled. Of these, *Aspergillus glaucus* and *A. candidus* have made up from 60% to 90% of the counts in the majority of samples, regardless of the location of the mill, the type of wheat milled,

TABLE 6  
MOLDS ISOLATED FROM FLOUR

- A. Prevalent in most samples  
*Aspergillus glaucus*  
*A. candidus*
- B. Prevalent in occasional samples  
*Penicillium* sp.  
*Aspergillus flavus*
- C. Present in small numbers in some samples  
*Aspergillus ochraceus*  
*A. niger*  
*A. versicolor*  
*A. terreus*  
*Rhizopus nigricans*  
*Mucor racemosus*  
*Hormodendrum* sp.  
*Alternaria tenuis*  
*Helminthosporium* sp.  
*Fusarium* sp.

or the time the samples were collected. In a few cases, species of *Penicillium* not yet identified have predominated. Other molds have been present only in small numbers, and then usually only in streams such as the purifier suction, where some contamination from outside air might be expected. The principal molds present in nearly all of the several hundreds of different samples we have assayed are those known to grow and sporulate at relative humidities between 75% and 85%. Thus they could be expected to prevail within most milling systems to a greater or less extent. The approximate composition of the fungus flora isolated by the writers from wheat flour is given in Table 6.

*Longevity of Fungi in Flour.* One sample of flour collected originally in a sterile tin container and one collected in a sterile paper bag were stored in the laboratory at room temperature for two to three

TABLE 7  
MOLD COUNTS OF FLOUR AFTER STORAGE FOR 2 TO 3 YEARS

| Sample           | Date tested | Molds per g. | Composition of fungus flora                                                        |
|------------------|-------------|--------------|------------------------------------------------------------------------------------|
| 1. Bakers Patent | Feb. '46    | 3,600        | <i>Aspergillus glaucus</i> 75%<br><i>A. candidus</i> 15%<br><i>Penicillium</i> 10% |
|                  | Sept. '47   | 2,700        | Same as in 1946                                                                    |
|                  | Jan. '49    | 1,400        | Same as in 1946                                                                    |
|                  | Feb. '47    | 7,200        | Principally <i>Aspergillus candidus</i>                                            |
| 2. 4th Break     | Jan. '49    | 7,200        | Same as in 1947                                                                    |

years, and periodically assayed for molds. The original and subsequent mold populations of these are given in Table 7.

Obviously the mold spores present in these flour samples lost their viability rather slowly. This perhaps is to be expected, since the spores of some of these molds are known to remain viable, in dry materials, for some years.

### Discussion

The data gathered in this study indicate that commercial flours generally are contaminated with from several hundred to several thousand viable mold spores per g., or from 100,000 to several million per pound. The flour from some mills appears to have a consistently higher mold count than that from other mills. The mold count of flour from a given mill seems to be determined more by conditions that prevail within the milling machinery than by the wheat from which the flour is milled. There is, at present, no evidence to indicate whether any of the molds found in the present study, in the numbers they were found, have any significant effect on flour quality. In some of the flours, considerable numbers of unidentified yeasts were present. The work of James and Smith (3) indicates that flours may contain various kinds of bacteria and yeasts as well as molds. Eventually it would seem desirable to determine whether the microfloral population of flour—bacteria, yeasts, and fungi—might, at times, affect the quality of flour for baking purposes.

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## COMMUNICATION TO THE EDITOR

### Preparation of Spray Dried Wheat Gluten<sup>1</sup>

DEAR SIR:

Drying of wheat gluten to preserve its bread baking qualities may be accomplished by exposure of the gluten in films or small pieces to air at room temperature, by evaporation of water from the frozen material at low pressures (lyophilization), or by vacuum oven drying at temperatures of 30–40°C. Of these methods, only the last is applicable to commercial operations at present and low capacity and long drying periods make it of limited value.

The finding that wheat gluten can be readily dispersed in 0.01 *N* acetic acid to give solutions containing up to 15% dry matter<sup>2</sup> suggested the possibility of spray drying these solutions to recover the gluten.

Gluten was dispersed in 0.01 *N* acetic acid, centrifuged to remove starch, precipitated by neutralizing the acetic acid and redispersed in 0.01 *N* acetic acid to give a concentration of approximately 12%.<sup>2</sup> The pH of the dispersions varied between 5.6–5.8 depending on the amount of gluten dispersed. The gluten dispersions were delivered to a laboratory spray drier of the conventional cyclone type described in detail elsewhere.<sup>3</sup> The spray in this small drier was produced by pumping the dispersion with a small centrifugal pump to the spray nozzle where it was atomized by an air jet operating at 20 p.s.i. The hot air used for drying was supplied at the rate of 90 cu. ft. per min. Inlet temperatures were varied from 250°F. (121°C.) to 300°F. (149°C.) (higher temperatures could not be obtained with the heating equipment available), and exhaust temperatures were varied from 175°F. (79°C.) to 225°F. (107°C.) (lower exhaust temperatures failed to dry material adequately). Within the range of air inlet and exhaust temperatures found practicable the gluten was recovered as a fine powder of about 2.5% moisture content. Most of the gluten was collected from the main drying cone but, owing to its low density, a considerable proportion was carried over and recovered from the collecting system.

<sup>1</sup> N.R.C. No. 2089.

<sup>2</sup> Lusena, C. V. Preparation of dried native wheat gluten. *Cereal Chem.* 27: 167–178, 1950

<sup>3</sup> Woodcock, A. H. and Tessier, H. A laboratory spray drier. *Can. J. Research, F.* 21: 75–77, 1943.

On addition of water the dried gluten immediately formed a sticky cohesive mass similar in physical characteristics to the original gluten. Retention of native properties in the dried gluten was assessed by solubility in dilute acetic acid solution and by baking tests. All gluten samples had 95% solubility in 0.005 *N* acetic acid indicating that little denaturation had taken place. The baking test consisted of addition of the gluten to a soft white spring wheat (containing 8.3% protein, 14% moisture basis) to raise the protein content to 13.5% and baking by the malt phosphate formula.<sup>4</sup>

TABLE I  
BAKING QUALITY OF WHEAT GLUTEN PREPARED BY SPRAY DRYING  
UNDER VARIOUS CONDITIONS  
(Gluten dispersions used contained approximately 12% dry matter)

| Sample no.                 | Operating conditions |              |                       | Baking quality    |        |         |
|----------------------------|----------------------|--------------|-----------------------|-------------------|--------|---------|
|                            | Drying temp., °F.    |              | Dried product, g./hr. | Loaf volume, c.c. | Crumb* |         |
|                            | Inlet                | Exhaust      |                       |                   | Color  | Texture |
| Control (air-dried gluten) | —                    | —            | —                     | 680               | 4.5    | 4.5     |
| Vac-ice dried gluten       | —                    | —            | —                     | 640               | 5.0    | 5.5     |
| Spray dried glutens        |                      |              |                       |                   |        |         |
| 1                          | 300 (149°C.)         | 175 (79°C.)  | 396                   | 680               | 5.5    | 5.5     |
| 2                          | 300 (149°C.)         | 200 (93°C.)  | 274                   | 640               | 5.0    | 6.0     |
| 3                          | 300 (149°C.)         | 225 (107°C.) | 209                   | 575               | 4.5    | 5.0     |
| 4                          | 275 (135°C.)         | 175 (79°C.)  | 274                   | 640               | 5.5    | 6.0     |
| 5                          | 275 (135°C.)         | 200 (93°C.)  | 187                   | 615               | 5.0    | 5.5     |
| 6                          | 250 (121°C.)         | 200 (93°C.)  | 122                   | 600               | 5.0    | 5.0     |

\* The Grain Research Laboratory, Winnipeg, uses the following verbal descriptions for both color and texture: excellent, 8.6–10.0; very good, 7.0–8.5; good, 5.5–6.9; fair, 4.5–5.4; poor, below 4.5.

Conditions for the drying operation and results of the baking tests are given in Table I. They show that loaf volume decreased with increasing exhaust temperatures at both the 300°F. (149°C) and 275°F. (135°C.) inlet temperatures. None equalled the control in loaf volume, but this is attributed to the use of 0.01 *N* acetic acid for dispersing the original gluten as it was later shown that 0.005 *N* acid gave large loaf volumes,<sup>3</sup> equivalent to those obtained with gluten preparations dried from the frozen state. An inlet air temperature of 300°F. (149°C.) and exhaust temperature of 175°F. (79°C.) gave the highest drying capacity and a slightly superior product.

<sup>4</sup> Aitken, T. R., and Geddes, W. F. The effect on flour strength of increasing the protein content by the addition of dried gluten. *Cereal Chem.* 15: 181–196 (1938).

While starch-free gluten was used in the present study, a crude gluten containing 10–15% starch could be used. These results show the feasibility of spray drying solutions of wheat gluten to yield a dry product that retains its bread baking properties.

November 8, 1949

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## Cereal Chemistry

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*Cereal Chemistry* publishes scientific papers dealing with raw materials, processes, or products of the cereal industries, or with analytical procedures, technological tests, or fundamental research, related thereto. Papers must be based on original investigations, not previously described elsewhere, which make a definite contribution to existing knowledge.

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**General.** From January 1, 1948, an abstract will be printed at the beginning of each paper instead of a summary at the end, references will be numbered to provide the option of citing by number only, and date of receipt, author's connections, etc., will be shown in footnotes. Except on these points, authors will find the last volume of *Cereal Chemistry* a useful guide to acceptable arrangements and styling of papers. "On Writing Scientific Papers for Cereal Chemistry" (*Trans. Am. Assoc. Cereal Chem.* 6: 1-22, 1948) amplifies the following notes.

Authors should submit two copies of the manuscript, typed double spaced with wide margins on 8½ by 11 inch white paper, and all original drawings or photographs for figures. If possible, one set of photographs of figures should also be submitted. Originals can then be held to prevent damage, and the photographs can be sent to reviewers.

**Titles and Footnotes.** Titles should be specific, but should be kept short by deleting unnecessary words. The title footnote shows "Manuscript received . . ." and the name and address of the author's institution. Author footnotes, showing position and connections, are desirable although not obligatory.

**Abstract.** A concise abstract of about 200 words follows title and authors. It should state the principal results and conclusions, and should contain, largely by inference, adequate information on the scope and design of the investigation.

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References are arranged and numbered in alphabetical order of authors' names and show author, title, journal, volume, first and last pages, and year. The list is given at the end of the paper. Reference numbers must invariably be cited in the text, but authors' names and year may be cited also. Abbreviations for the names of journals follow the list given in *Chemical Abstracts* 40: I-CCIX, 1946.

**Organization.** The standard organization involves main sections for abstract, introduction, materials, methods, results, discussion, acknowledgments, and literature cited. Alternately, a group of related studies, each made with different materials or methods, may require a separate section for each study, with subsections for materials and methods, and for results, under each section. Center headings are used for main sections and italicized run-in headings for subsections, and headings should be restricted to these two types only.

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Tables should be typed on separate pages at the end of the manuscript, and their position should be indicated to the printer by typing "(TABLE I)" in the appropriate place between lines of the text. (Figures are treated in the same way.)

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Use names, not formulas, for text references to chemical compounds. Use plural verbs with quantities (6.9 g. were). Figures are used before unit abbreviations (3 ml.), and % rather than "per cent" is used following figures. All units are abbreviated and followed by periods, except units of time, which are spelled out. Repeat the degree sign ( $5^{\circ}$ - $10^{\circ}$  C.). Place 0 before the decimal point for correlation coefficients ( $r=0.95$ ). Use \* to mark statistics that exceed the 5% level and \*\* for those that exceed the 1% level; footnotes explaining this convention are no longer required. Type fractions on one line if possible, e.g.,  $A/(B+C)$ . Use lower case for farinograph, mixogram; etc., unless used with a proper name, i.e., Brabender Farinograph. When in doubt about a point that occurs frequently, consult the *Style Manual* or the *Dictionary*.

# CEREAL CHEMISTRY

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## AN INVESTIGATION OF COMMERCIAL FUNGAL AND BACTERIAL ALPHA-AMYLASE PREPARATIONS IN BAKING<sup>1</sup>

J. F. CONN,<sup>2</sup> J. A. JOHNSON,<sup>4</sup> and B. S. MILLER<sup>3</sup>

### ABSTRACT

Commercial alpha-amylase preparations including two bacterial and six fungal sources were employed as diastatic supplements and compared with malted wheat flour. It was demonstrated that fungal preparations may be used for commercial alpha-amylase supplementation if the ratio of proteinase to alpha-amylase is not excessive. The two bacterial preparations caused the bread crumb to be sticky and gummy and were, therefore, undesirable as flour supplements. These properties were associated with the amylase and not the proteinase activity. The reasons for the sticky and gummy bread crumb could not be fully determined but may be explained by the lesser affinity of the bacterial amylase enzyme for low molecular weight dextrans. Separate proteinase and alpha-amylase supplementation studies indicated that hard red spring wheat flours may require more proteinase than hard red winter wheat flours to produce optimum bread. Both alpha-amylase and proteinase supplementation increased the bread crumb compressibility.

The proteinase and alpha-amylase activities of a flour supplemented with a fungal enzyme concentrate decreased during storage at various temperatures. Lower temperatures favored greater retention of enzymatic activity. The rate of decrease was approximately the same whether the supplemented flour was stored in the presence of air, nitrogen, or oxygen. Bacterial spore counts and bread storage data indicated no significant difference in degree of contamination at equivalent levels of supplementation by suitable commercial fungal alpha-amylase preparations and the supplements now in use.

The ability of malted cereals to impart desirable characteristics to bread has been long recognized. In recent decades the desirable characteristics have been shown to result from the action of certain

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enzyme systems which are present in the malted grain. Germinated cereals are the most common source of diastatic supplements. Other possible sources include those produced by culturing bacteria and fungi. There has been extensive commercial production of alpha-amylase by the growth of selected strains of *Bacillus subtilis*, *Bacillus mesentericus*, and *Aspergillus oryzae* on suitable media. The amylases generally produced by these organisms have been found to be of the alpha type. Green (4), Kneen and Sandstedt (10), and Miller and Johnson (13) suggest that fungal alpha-amylase may be suitable for use in baking.

The evaluation of alpha-amylase sources for baking purposes is not a simple problem. Kozmin (11), and Proskuryakov, Grinberg and Kozhevnikova (15) have shown that excessive malt alpha-amylase supplementation caused inelastic and sticky crumb. Miller and Johnson (13), however, observed no stickiness of the crumb when using an aqueous malt extract equivalent to 6% malted wheat flour. Bacterial amylases have shown little promise as diastatic supplements (7) due to the production of sticky bread crumb. Kneen and Sandstedt (10) reported that bacterial amylases have considerably higher thermostability than malt alpha-amylase. Hopkins and Kulka (5) suggested that the differences in the behavior of bacterial and malt alpha-amylase may be due to less affinity of bacterial alpha-amylase for low grade dextrans. Both of these properties could explain the excessive starch liquefaction due to bacterial alpha-amylase resulting in sticky bread crumb characteristics.

Enzymes other than alpha-amylase are also of concern in supplementation. The improvement in loaf volume and grain resulting from the addition of small amounts of several proteolytic preparations to flours was attributed by Read and Haas (16) to a mellowing action on the gluten, giving a more workable dough. Excessive dosages of proteinases have been shown to be detrimental by many workers (3, 7, 9, 13, 16). Miller and Johnson (14) developed technics appropriate for the inactivation of either alpha-amylase or proteinase in malted wheat and barley flour and fungal preparations. Johnson and Miller (9) have studied the role of alpha-amylase and proteinase in bread-making. These workers (9) found that high concentrations of proteinase provided by a fungal preparation caused detrimental effects to loaf volume, grain and texture. Fungal preparations were shown to be satisfactory as diastatic supplements if the ratio of amylase and proteinase is controlled.

While present regulations in the United States permit flour to be supplemented only with malted wheat or barley flour, hearings have been held recently by the Federal Security Agency preparatory to

issuing new "standards of identity" for bread. One subject for consideration has been the use of alpha-amylase from sources other than malted wheat or barley.

The objective of this investigation was to study the characteristics of commercial bacterial and fungal alpha-amylase preparations and to determine the feasibility of their commercial use as alpha-amylase supplements. Consideration also was given to the retention of alpha-amylolytic and proteolytic activity in flours supplemented with a commercial fungal alpha-amylase preparation and stored under controlled conditions.

### Materials and Methods

A commercial hard red winter, straight grade flour, having a protein content of 11.8% (14.0% moisture basis), was used for the majority of the experiments. In addition, three hard red spring and two hard red winter bakers' patent flours ranging in protein content from 11.5% to 12.5% were used in studying the separate effects of alpha-amylase and proteinase supplementation. All flours were unmalted and showed good malt response.

The alpha-amylase supplements included a commercial malted wheat flour and eight commercial enzyme concentrates, six of which were of fungal and two of bacterial origin (Table I).

TABLE I  
ALPHA-AMYLASE AND PROTEINASE ACTIVITIES OF VARIOUS ENZYME  
PREPARATIONS COMPARED TO MALTED WHEAT FLOUR

| Enzyme                          | Ratios of activities |            |                                      |
|---------------------------------|----------------------|------------|--------------------------------------|
|                                 | Alpha amylase        | Proteinase | Ratio of proteinase to alpha-amylase |
| Malted wheat flour <sup>1</sup> | 1                    | 1          | 1                                    |
| Maltase-20 (fungal)             | 66                   | 67         | 1                                    |
| Rhozyme-S (fungal)              | 120                  | 1000       | 8.3                                  |
| Diastase-29 (fungal)            | 0.21                 | 417        | 2000                                 |
| Diastase-32 (fungal)            | 5.7                  | 244        | 43                                   |
| Diastase-33 (fungal)            | 50                   | 31.6       | 0.63                                 |
| Diastase-34 (fungal)            | 80                   | 0.19       | 0.002                                |
| Diastase-28 (bacterial)         | 31                   | 5.7        | 0.18                                 |
| Diastase-30 (bacterial)         | 24                   | 69         | 2.9                                  |

<sup>1</sup> 40 alpha-amylase units of activity (17) per gram.

The sponge-dough baking procedure reported by Johnson and Miller (9) was employed. Alpha-amylase dextrinization activity was determined by the procedure described by Sandstedt, Kneen and Blish (17).<sup>4</sup> The starch liquefying activity of the alpha-amylase was de-

<sup>4</sup> Although the method used for determining dextrinogenic activity was designed for malt preparations, it has also been used in this study for determining the dextrinogenic activity of both fungal and bacterial amylases.



terminated with the amylograph employing 65 gms. of flour and 450 ml. of liquid as described by Anker and Geddes (2). Proteinase activity was determined by the Ayre-Anderson method as modified by Miller (12).

Arbitrary terms were defined to indicate the proteinase and alpha-amylase concentrations used in baking. The term "1X" indicates alpha-amylase supplementation equivalent to the alpha-amylase added by 0.25% malted wheat flour having an activity of 40 alpha-amylase units per gm. The term "1Y" indicates a level of proteinase supplementation per 100 g. of flour equal to that amount of proteinase which would give a titration of one ml. of 0.0714 *N* sodium hydroxide in the proteolytic activity determination (12).

The effect of temperature on the inactivation of alpha-amylase from various sources was determined by the technique used by Johnson and Miller (8). Techniques used for the differential inactivation of alpha-amylase and proteinase were described by Miller and Johnson (14).

Crumb compressibility was determined with a Bloom Gelometer after storage of the bread in sealed plastic bags for periods of 24 and 96 hours. The experimental value was expressed in grams of lead shot required to press a one-inch plunger 4 mm. into a slice of bread. Two determinations on each of two slices cut from three loaves chosen from each experimental group were recorded.

The effect of oxygen, nitrogen, and air atmospheres on enzyme activity was studied by storing flour supplemented with Rhozyme-S, one of the fungal preparations, at a level equivalent to 1% malted wheat flour alpha-amylase. The samples were stored at 5°C., room temperature, and 35°C. for eight months.

The A.A.C.C. method (1) for determining the total bacterial spores in flour was used for determination of spore counts in the various enzyme preparations. James and Smith (6) have indicated that this method gives only presumptive indications of the number of rope spores.

### Results and Discussion

The alpha-amylase activities of six fungal and two bacterial preparations were found to vary from a fraction to as much as 120 times that of the commercial malted wheat flour (Table I). The proteinase activities of these same enzyme concentrates were found to vary from a fraction to as much as 1,000 times that of malted wheat flour. The ratio of proteinase to alpha-amylase was found to vary from a fraction to as much as 2,000 times that of malted wheat flour.

Preliminary experimental sponge baking studies using quantities of these enzyme preparations which provided equivalent alpha-

amylase levels indicated that the bacterial preparations, Diastase-28 and -30, produced sticky bread crumb. These preparations produced no evidence of excessive proteolytic activity. Diastase-29 and Diastase-32, however, contained excessive amounts of proteinase as measured by chemical means and as indicated during fermentation. No sticky bread crumb was observed in bread containing these preparations. Rhozyme-S, Maltase-20, Diastase-33, and Diastase-34, as well as the two bacterial preparations were believed to warrant further investigation.

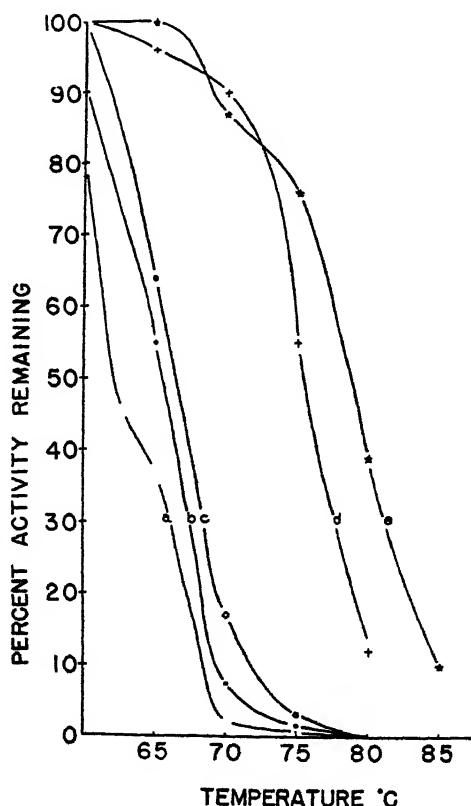


Fig. 1. The effect of heating and enzyme source on the retention of alpha-amylase activity; a, Diastase-28, bacterial; b, Diastase-30, bacterial; c, Rhozyme-S, fungal; d, Malted wheat flour; and e, Rhozyme-DX, bacterial.

*Bacterial Concentrates as Enzyme Supplements.* The evidence in the literature (7, 10) suggests that some bacterial alpha-amylases have high thermostability. The effect of temperature on the inactivation of alpha-amylase was studied, therefore, as one of the possible explanations of crumb stickiness resulting from the use of bacterial Diastase-28

and -30. The results (Fig. 1) obtained for various preparations including those for a bacterial preparation (Rhozyme-DX) known to possess high thermostability show that the alpha-amylases of Diastase-28 and -30 were actually less thermostable than malted wheat flour alpha-amylase. Rhozyme-DX, as expected, possessed high thermostability. These results showing that amylases from different bacterial strains may differ in thermostability corroborate the work of Tilden and Hudson (18). Thus, the thermostability of these bacterial alpha-amylases does not explain the reason for sticky crumb caused by these particular bacterial preparations.

The effect of elevated temperatures on the relative dextrinogenic activity of alpha-amylase from various sources was also investigated. The dextrinogenic activity of malted wheat flour at 50°C. as compared with that at 30°C. was found to increase more than that of Diastase-28

TABLE II  
EFFECT OF ALPHA-AMYLASES FROM VARIOUS SOURCES ON MAXIMUM  
AMYLOGRAPH CURVE HEIGHTS

| Preparation        | Concentration <sup>1</sup> | Maximum height    | Per cent height of corresponding malted wheat flour curve |
|--------------------|----------------------------|-------------------|-----------------------------------------------------------|
|                    |                            | B.U. <sup>2</sup> | %                                                         |
| Malted wheat flour | 1X                         | 385               | —                                                         |
| Malted wheat flour | 1/2X                       | 630               | —                                                         |
| Diastase-28        | 1X                         | 340               | 88                                                        |
| Diastase-28        | 1/2X                       | 550               | 87                                                        |
| Diastase-30        | 1X                         | 280               | 73                                                        |
| Diastase-30        | 1/2X                       | 480               | 76                                                        |

<sup>1</sup> 1X—concentration equivalent to the alpha-amylase provided by 0.25% malted wheat flour supplementation.

<sup>2</sup> B.U.—Brabender units.

and -30. This would suggest that the alpha-amylase of malted wheat flour would be more active at this temperature than the alpha-amylase of Diastase-28 and -30. Maximum amylograph viscosities obtained with malted wheat flour were higher than those obtained when equivalent levels of Diastase-28 and -30 were used (Table II). Thus, based on amylograph data, equivalent levels of Diastase-28 and -30 would be expected to produce greater starch degradation than an equivalent level of malted wheat flour. However, from the temperature of inactivation data and the dextrinization activities at the elevated temperature, malted wheat flour would be expected to produce greater starch degradation than the bacterial preparations. This apparent anomaly may be explained by assuming a lesser affinity of bacterial alpha-amylase for lower molecular weight dextrans as suggested by Hopkins and Kulka (5). Accordingly bacterial alpha-amylase mole-

cules may be free to split greater numbers of starch molecules, with a corresponding increase in dextrin formation and stickiness of bread crumb.

Crumb stickiness due to the presence of bacterial alpha-amylase was evident at 0.1X concentration of either Diastase-28 or -30. This stickiness was accentuated at higher concentrations. No dough stickiness associated with excessive proteolytic activity was observed at any time during fermentation. From the baking results (Table III) it was concluded that as the concentration of Diastase-28 or -30 was increased, sticky bread crumb resulted before any beneficial effects

TABLE III  
EFFECT OF STARCH LIQUEFYING AND SACCHARIFYING ENZYMES  
ON DEVELOPMENT OF CRUMB STICKINESS

| DIASTASE-28                          |                                             |       |         |             |                  |
|--------------------------------------|---------------------------------------------|-------|---------|-------------|------------------|
| Rhozyme-S concentration <sup>1</sup> | Bacterial enzyme concentration <sup>1</sup> | Grain | Texture | Loaf volume | Crumb properties |
| 0                                    | 0.04X                                       | %     | %       | cc.         | Satisfactory     |
| 0                                    | 0.1X                                        | 68    | 70      | 2835        | Slightly sticky  |
| 0                                    | 0.25X                                       | 65    | 65      | 2840        | Slightly sticky  |
| 1X                                   | 1X                                          | 67    | 65      | 2880        | Very sticky      |
| 4X                                   | 1X                                          | 50    | 50      | 3015        | Very sticky      |
|                                      |                                             | 55    | 50      | 2940        | Very sticky      |
| DIASTASE-30                          |                                             |       |         |             |                  |
| 0                                    | 0.04X                                       | 65    | 65      | 2950        | Satisfactory     |
| 0                                    | 0.1X                                        | 65    | 65      | 3060        | Slightly sticky  |
| 0                                    | 0.25X                                       | 60    | 65      | 2900        | Slightly sticky  |
| 1X                                   | 1X                                          | 55    | 50      | 3025        | Very sticky      |
| 4X                                   | 1X                                          | 50    | 50      | 2915        | Very sticky      |
| 1X                                   | 0                                           | 85    | 80      | 2975        | Satisfactory     |
| 4X                                   | 0                                           | 80    | 83      | 2925        | Satisfactory     |
| 0                                    | 0                                           | 70    | 75      | 2865        | Satisfactory     |

<sup>1</sup> 1X—concentration of alpha-amylase equivalent to the alpha-amylase provided by 0.25% malted wheat flour supplementation.

appeared. Thus, Diastase-28 and -30 were not suitable as diastatic supplements for baking.

*Fungal Concentrates as Enzyme Supplements.* An investigation was made of supplementation with Rhozyme-S, Maltase-20, Diastase-33 and -34 compared with malted wheat flour using alpha-amylase concentrations of 1X, 4X, and 8X. The results are shown in Table IV. Each fungal preparation was blended with flour to give a product equivalent in alpha-amylase activity to that of malted wheat flour. Optimum results were obtained with malted wheat flour at 4X concentration while Rhozyme-S and Maltase-20 produced best results at

TABLE IV  
BAKING DATA USING RHOZYME-S AND MALTASE-20, DILUTED WITH  
FLOUR AS ALPHA-AMYLASE SUPPLEMENTS

| Preparation        | Concentration <sup>1</sup> | Grain   | Texture | Loaf volume | Dough properties |
|--------------------|----------------------------|---------|---------|-------------|------------------|
| —                  | 0                          | %<br>80 | %<br>80 | cc.<br>2858 | Satisfactory     |
| Malted wheat flour | 1X                         | 90      | 90      | 2971        | Satisfactory     |
| Malted wheat flour | 4X                         | 92      | 90      | 3025        | Slightly slack   |
| Malted wheat flour | 8X                         | 85      | 85      | 3105        | Slightly slack   |
| Rhozyme-S          | 1X                         | 87      | 85      | 2992        | Slightly slack   |
| Rhozyme-S          | 4X                         | 80      | 83      | 2963        | Slack            |
| Rhozyme-S          | 8X                         | 70      | 80      | 2945        | Very slack       |
| Maltase-20         | 1X                         | 90      | 88      | 2942        | Satisfactory     |
| Maltase-20         | 4X                         | 88      | 88      | 3058        | Slightly slack   |
| Maltase-20         | 8X                         | 70      | 75      | 2975        | Slightly slack   |
| Diastase-33        | 1X                         | 88      | 88      | 2947        | Satisfactory     |
| Diastase-33        | 8X                         | 90      | 90      | 2920        | Slightly slack   |
| Diastase-34        | 1X                         | 88      | 90      | 3020        | Satisfactory     |
| Diastase-34        | 8X                         | 92      | 92      | 3060        | Satisfactory     |

<sup>1</sup> 1X—concentration equivalent to the alpha-amylase provided by 0.25% malted wheat flour supplementation.

1X concentration. The bread baked with Diastase-33 and -34 did not exhibit a marked optimum alpha-amylase level, but appeared to be adequately supplemented at 1X concentration. Increasing the concentration of Rhozyme-S and Maltase-20 beyond the 1X level caused the grain and texture to become inferior. There was no evidence of sticky or gummy crumb characteristics at these high alpha-amylase concentrations. The dough, however, slackened excessively during

TABLE V  
BAKING DATA USING RHOZYME-S WITH VARIOUS PERCENTAGES  
OF THE PROTEINASE REMOVED

| Alpha-amylase concentration <sup>1</sup> | Proteinase retained | Grain   | Texture | Loaf volume | Dough properties |
|------------------------------------------|---------------------|---------|---------|-------------|------------------|
| 0                                        | %<br>—              | %<br>70 | %<br>70 | cc.<br>2833 | Satisfactory     |
| 1X                                       | 10                  | 85      | 90      | 2916        | Satisfactory     |
| 1X                                       | 25                  | 75      | 85      | 2796        | Satisfactory     |
| 1X                                       | 50                  | 80      | 80      | 2696        | Slightly slack   |
| 1X                                       | 75                  | 75      | 75      | 2879        | Slack            |
| 4X                                       | 10                  | 85      | 90      | 3008        | Satisfactory     |
| 4X                                       | 25                  | 83      | 88      | 2971        | Satisfactory     |
| 4X                                       | 50                  | 78      | 85      | 2946        | Slack            |
| 4X                                       | 75                  | 75      | 75      | 2879        | Slack            |

<sup>1</sup> 1X—concentration equivalent to the alpha-amylase provided by 0.25% malted wheat flour.

fermentation due to excessive proteolysis and became difficult to machine when the highest enzyme concentrations were employed.

Baking results obtained with Rhozyme-S from which various proportions of the total proteinase activity had been removed are presented in Table V. A tendency to produce slack dough was noted when 50% of the proteinase was present and considerable slackening was observed at both 1X and 4X concentrations of alpha-amylase containing three-fourths of the original proteinase activity. The best

TABLE VI  
BAKING DATA INDICATING THE SEPARATE EFFECTS OF ALPHA-AMYLASE  
AND PROTEINASE SUPPLEMENTATION

| Rhozyme-S<br>alpha-amylase<br>concentration <sup>1</sup> | Diastase-29<br>proteinase<br>concentration <sup>2</sup> | Grain | Texture | Loaf volume | Dough properties |
|----------------------------------------------------------|---------------------------------------------------------|-------|---------|-------------|------------------|
| HARD RED WINTER FLOUR                                    |                                                         |       |         |             |                  |
| 0                                                        | 0                                                       | 75    | 80      | 2916        | Satisfactory     |
| 0                                                        | 1Y                                                      | 80    | 78      | 2981        | Satisfactory     |
| 0                                                        | 4Y                                                      | 85    | 80      | 2988        | Slightly slack   |
| 1X                                                       | 0                                                       | 90    | 88      | 3059        | Satisfactory     |
| 1X                                                       | 1Y                                                      | 90    | 88      | 3041        | Slightly slack   |
| 1X                                                       | 4Y                                                      | 88    | 85      | 2959        | Slack            |
| 8X                                                       | 0                                                       | 90    | 90      | 3062        | Satisfactory     |
| 8X                                                       | 1Y                                                      | 88    | 90      | 3066        | Slightly slack   |
| 8X                                                       | 4Y                                                      | 85    | 87      | 3056        | Slack            |
| HARD RED SPRING FLOUR                                    |                                                         |       |         |             |                  |
| 0                                                        | 0                                                       | 75    | 80      | 2788        | Slightly bucky   |
| 0                                                        | 1Y                                                      | 77    | 82      | 2738        | Satisfactory     |
| 0                                                        | 4Y                                                      | 77    | 82      | 2869        | Satisfactory     |
| 1X                                                       | 0                                                       | 80    | 85      | 2747        | Satisfactory     |
| 1X                                                       | 1Y                                                      | 85    | 87      | 2975        | Satisfactory     |
| 1X                                                       | 4Y                                                      | 92    | 90      | 2994        | Slightly slack   |
| 8X                                                       | 0                                                       | 80    | 87      | 2888        | Satisfactory     |
| 8X                                                       | 1Y                                                      | 92    | 90      | 2953        | Slightly slack   |
| 8X                                                       | 4Y                                                      | 92    | 90      | 2959        | Slightly slack   |

<sup>1</sup> 1X—concentration of alpha amylase equivalent to the alpha-amylase provided by 0.25% malted wheat flour supplementation.

<sup>2</sup> 1Y—the addition to 100 grams of flour of that amount of Diastase-29 proteinase which would give a titration of one ml. in a proteolytic activity determination.

bread was obtained when only 10% to 25% of the original proteinase was present. These results demonstrated the desirability of reducing the proteinase activity of Rhozyme-S.

The data in Table V were further substantiated by studies in which the alpha-amylase and proteinase were added as separate adjuncts. Rhozyme-S having up to 98% of the proteinase activity removed was used in combination with small amounts of the proteinase derived from

the same preparation by appropriate differential inactivation techniques. Both external and internal characteristics indicated that 4X and 8X concentration of the alpha-amylase provided optimum amylase supplementation for a hard red winter wheat flour. Some improvement in quality of the bread was observed for proteinase supplementation equivalent to that provided by 1X concentration of the original preparation.

TABLE VII  
EFFECT OF ENZYME SOURCE ON BREAD CRUMB COMPRESSIBILITY

| Enzyme source      | Concentration | Compressibility of bread stored <sup>1</sup> |             |
|--------------------|---------------|----------------------------------------------|-------------|
|                    |               | 24 hours                                     | 96 hours    |
| Control            | 0             | g.<br>98.8                                   | g.<br>126.8 |
| Malted wheat flour | X             | 89.6                                         | 113.1       |
| Malted wheat flour | 4X            | 94.5                                         | 98.3        |
| Rhozyme-S          | X             | 92.7                                         | 94.8        |
| Rhozyme-S          | 4X            | 79.4                                         | 100.4       |
| Maltase-20         | X             | 84.5                                         | 88.7        |
| Maltase-20         | 4X            | 79.0                                         | 85.8        |

ANALYSIS OF VARIANCE

| Source of variation      | Degrees of freedom | Mean square |
|--------------------------|--------------------|-------------|
| Enzyme concentration     | 1                  | 1290**      |
| Days of storage          | 1                  | 5100***     |
| Enzyme source            | 2                  | 1717***     |
| Concentration X storage  | 1                  | 142         |
| Concentration X source   | 2                  | 279*        |
| Storage X source         | 2                  | 525*        |
| Conc. X storage X source | 2                  | 613**       |
| Within source            | 132                | 120         |

<sup>1</sup> Average of twelve separate readings. The grams of lead shot required to press a one-inch plunger 4 mm. into bread crumb one inch thick.

\* Significance exceeds 5% level.

\*\* Significance exceeds 1% level.

\*\*\* Significance exceeds 0.1% level.

$\bar{x}_{.01} = 11.68$ —the difference between compressibility values for different enzyme sources required for significance at the 1% level.

$\bar{x}_{.05} = 8.84$ —the difference between compressibility values for different enzyme sources required for significance at the 5% level.

Further investigations concerning the separate effects of alpha-amylase and proteinase were performed using Diastase-29, proteinase, and Rhozyme-S from which the proteinase had been removed as the source of alpha-amylase. Baking experiments were performed using three commercially milled hard red winter and three commercially milled hard red spring wheat flours with various combinations of these enzymes. Typical results are shown in Table VI. Optimum bread for the hard red winter flour was obtained when no proteinase was

added but the flour easily tolerated proteinase up to 1Y concentration. It was concluded that optimum quality bread was obtained when the hard red winter wheat flours under investigation received 0 to 0.5Y proteinase supplementation.

Optimum quality bread was obtained with the hard red spring wheat flour when 1 to 4Y concentration of Diastase-29 was used. The results obtained for two additional flours also indicated that at least 1Y concentration of Diastase-29 was required for optimum proteinase supplementation. There appeared to be little difference between 1X and 8X alpha-amylase supplementation. The results obtained indicate that the optimum level of proteinase supplementation for hard red spring wheat flours is substantially greater than the optimum level for hard red winter wheat flours.

TABLE VIII  
EFFECT OF FUNGAL ALPHA-AMYLASE AND PROTEINASE SUPPLEMENTATION  
ON BREAD CRUMB COMPRESSIBILITY

| Alpha-amylase<br>concentration <sup>1</sup> | Proteinase<br>concentration <sup>2</sup> | Compressibility of bread stored |                        |
|---------------------------------------------|------------------------------------------|---------------------------------|------------------------|
|                                             |                                          | 24 hours                        | 96 hours               |
|                                             |                                          | <i>g.</i> <sup>3</sup>          | <i>g.</i> <sup>3</sup> |
| 0                                           | 0                                        | 100.4                           | 152.0                  |
| 0                                           | 1Y                                       | 95.1                            | 149.3                  |
| 0                                           | 4Y                                       | 85.8                            | 144.4                  |
| 1X                                          | 0                                        | 83.9                            | 131.6                  |
| 1X                                          | 1Y                                       | 82.3                            | 125.3                  |
| 1X                                          | 4Y                                       | 74.6                            | 120.5                  |
| 8X                                          | 0                                        | 82.2                            | 126.6                  |
| 8X                                          | 1Y                                       | 75.8                            | 120.4                  |
| 8X                                          | 4Y                                       | 71.0                            | 118.7                  |

<sup>1</sup> 1X—concentration of alpha-amylase equivalent to the alpha-amylase provided by 0.25% malted wheat flour supplementation.

<sup>2</sup> 1Y—concentration, the addition to 100 grams of flour of that amount of Diastase-29 proteinase which would give a delta titration of 1 ml. in the proteolytic activity determination.

<sup>3</sup> The grams of lead shot required to press a one-inch plunger 4 mm. into bread crumb, one inch thick. Average of twelve separate readings.

A comparison of the effects of malted wheat flour, Rhozyme-S and Maltase-20 on the compressibility of bread is shown in Table VII. The analysis of variance for this data also is included in Table VII. Enzyme source and concentration, as well as length of storage, affected the compressibility significantly. After 24 hours of storage, the bread baked with 4X concentration of Rhozyme-S, 1X and 4X concentration of Maltase-20 was significantly more compressible than bread containing no enzyme supplement. After 96 hours of storage, all enzyme treatments produced bread which was significantly more compressible than the control bread. At X concentration of alpha-amylase, bread baked with either Rhozyme-S or Maltase-20 was significantly more



compressible than the bread baked with a corresponding concentration of alpha-amylase from malted wheat flour.

The separate effects of alpha-amylase and proteinase supplementation on bread crumb compressibility are illustrated in Table VIII. Data were obtained after 24 and 96 hours of storage. The analysis of variance of these data is shown in Table IX. Both alpha-amylase and proteinase increased, while storage decreased the crumb compressibility. From calculations of least significant mean differences, it was determined that 4Y concentration of proteinase was required to produce a crumb compressibility significantly greater than that of the crumb of bread containing no added proteinase. The bread crumb of loaves baked with either 1X or 8X concentration of alpha-amylase was significantly more compressible than the crumb of loaves containing no added alpha-amylase. No significant differences in compressibility,

TABLE IX  
ANALYSIS OF VARIANCE OF THE SEPARATE EFFECT OF ALPHA-AMYLASE,  
PROTEINASE, AND STORAGE ON BREAD CRUMB COMPRESSIBILITY

| Source of variation                  | Degrees of freedom | Mean square |
|--------------------------------------|--------------------|-------------|
| Proteinase                           | 2                  | 1,926**     |
| Alpha-amylase                        | 2                  | 9,988**     |
| Storage                              | 1                  | 127,653**   |
| Proteinase X alpha-amylase           | 4                  | 26          |
| Proteinase X storage                 | 2                  | 57          |
| Alpha-amylase X storage              | 2                  | 517**       |
| Proteinase X alpha-amylase X storage | 4                  | 35          |
| Individual values                    | 198                | 119         |
| Total                                | 215                |             |

\*\* Significant at the 1% level.

however, were observed between 1X and 8X alpha-amylase concentration. Combinations of alpha-amylase and proteinase produced bread crumb with greater compressibility. It would appear that the choice of alpha-amylase level might not be so critical as that of the proteinase level.

*Stability During Storage of Proteinase and Alpha-Amylase in Rhozyme-S Supplemented Flour.* The effects of storage on the retention of alpha-amylase and proteinase activity of flours stored under oxygen, nitrogen, and air atmosphere at three different temperatures are shown in Fig. 2. The analyses of variance of these data are presented in Table X. Significant differences in both alpha-amylase and proteinase as affected by temperature and length of storage were observed. The lower storage temperature was favorable to the retention of both alpha-amylase and proteinase. The effect of storage under various gases was similar.

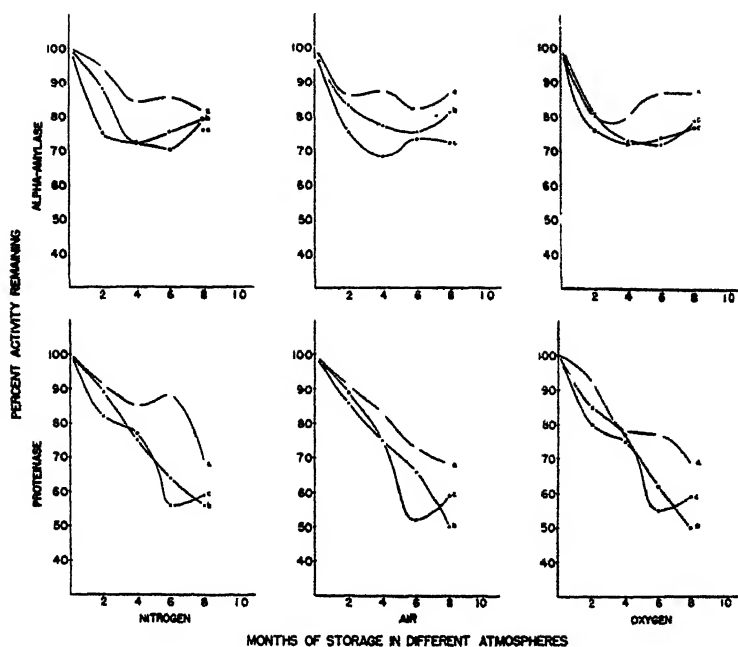


Fig. 2. The effect of gaseous atmosphere, temperature and length of storage on the retention of alpha-amylase and proteinase activities in flour supplemented with Rhozyme-S; a, 5°C; b, room temperature; and c, 35°C.

*Microflora of Enzyme Preparations.* The number of bacterial spores per gram of preparation was determined for various enzyme concentrates suitable for supplementation. The counts for Rhozyme-S

TABLE X  
ANALYSES OF VARIANCE OF THE EFFECT OF TEMPERATURE AND GASEOUS  
ATMOSPHERE UPON THE RETENTION OF ALPHA-AMYLASE  
AND PROTEINASE ACTIVITY DURING STORAGE

| Source of variation                      | Degrees of freedom | Mean square              |                       |
|------------------------------------------|--------------------|--------------------------|-----------------------|
|                                          |                    | Alpha-amylase activities | Proteinase activities |
| Atmosphere                               | 2                  | 5                        | 19                    |
| Temperature                              | 2                  | 315**                    | 413**                 |
| Length of storage                        | 4                  | 857**                    | 2752**                |
| Atmosphere×temperature                   | 4                  | 7                        | 9                     |
| Atmosphere×length of storage             | 8                  | 10                       | 6                     |
| Temperature×length of storage            | 8                  | 29                       | 76**                  |
| Atmosphere×length of storage×temperature | 16                 | 8                        | 13                    |
| Total                                    | 44                 |                          |                       |

\*\* Significant at the 1% level.

TABLE XI  
BACTERIAL SPORE COUNTS FOR VARIOUS PREPARATIONS  
SUITABLE AS SUPPLEMENTS

| Preparation        | Bacterial spores per gram | No. of spores added to 100 g. of flour when supplemented to equivalent levels |
|--------------------|---------------------------|-------------------------------------------------------------------------------|
| Malted wheat flour | 2,100                     | 500 <sup>1</sup>                                                              |
| Rhozyme-S          | 405,000                   | 800 <sup>1</sup>                                                              |
| Maltase-20         | 1,000,000                 | 10 <sup>1</sup>                                                               |
| Diastase-29        | 2,500                     | 2,300 <sup>2</sup>                                                            |

<sup>1</sup> Supplemented to 1X alpha-amylase concentration

<sup>2</sup> Supplemented to 1Y proteinase concentration.

and Diastase-29 appear very high (Table XI). However, based on equivalent alpha-amylase levels of supplementation there was little difference between Rhozyme-S, Maltase-20, and malted wheat flour. Rope development was not observed in bread baked with any of these preparations and stored up to 8 days under conditions favorable for mold growth. Commercial enzyme preparations may be prepared having considerably lower spore counts if desired.

#### Acknowledgment

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## FACTORS AFFECTING THE COLOR OF MACARONI

### II. KINETIC STUDIES OF PIGMENT DESTRUCTION DURING MIXING <sup>1</sup>

G. N. IRVINE <sup>2</sup> and C. A. WINKLER <sup>3</sup>

#### ABSTRACT

The destruction of the xanthophyll pigments during the mixing stage of macaroni processing has been followed kinetically under a variety of mixing conditions. The rate increases with increasing absorption to a maximum at about 33%; with increasing temperature; and with increasing oxygen concentration of the mixing atmosphere. The reaction is inhibited by thousandth molar cyanide and alpha naphthol and by alcohol concentrations above 10%. Reversal of the reaction occurs on prolonged mixing in 40% alcohol. Evidence is provided to support the hypothesis that the destruction of pigment occurs through a coupled reaction involving the peroxidation of unsaturated fat by the enzyme lipoxidase. It is proposed that this enzyme system functions, in semolina, in conjunction with an activating enzyme which is inhibited by cyanide and accelerated by some heavy metal ions. A mechanism is proposed for the reaction which accounts for the phenomena observed.

Most varieties of durum wheat grown in North America yield macaroni which is physically satisfactory, handles well, and has good

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cooking quality. Hence the differentiation of good and poor varieties for macaroni-making purposes is made largely on the basis of the color of the macaroni made therefrom. This should be a bright yellow or yellow amber color.

Macaroni with a high yellow pigment content can be obtained only from wheat that is high in this pigment, and plant breeders are constantly striving to produce such wheats. This factor in itself, however, does not necessarily ensure a good yellow macaroni. There are several older varieties, such as Golden Ball and Pelissier, which have a characteristically high yellow pigment content, but which produce macaroni that loses a great deal of pigment during processing. It has been found in this laboratory that the percentage of pigment destroyed during processing varies from about 20% for the best varieties to about 60% for the poorest varieties. Destruction has been shown to occur principally during mixing, though a small additional loss occurs during extrusion. This paper describes a kinetic study, dealing with the decrease in pigment with time under a variety of mixing conditions, that serves to elucidate the principal factors and mechanisms involved in destruction of the yellow pigments.

### Materials and Methods

Samples of a number of pure varieties covering a wide range of macaroni-making quality were used in the investigation. Semolinas were milled to 50% yield on an Allis-Chalmers laboratory mill.

The semolinas were processed by the disc method of Cunningham and Anderson (5) which involves preparing doughs from 50 g. samples in a micro mixer. This mixer, because of its small scale and special design, is much more severe than commercial mixers; it produces a cohesive and relatively uniform dough in about  $1\frac{1}{4}$  minutes, as compared with 10 to 15 minutes for commercial models. Thus mixing times reported in this paper are not directly comparable with those obtained in commercial plants.

Studies with the micro macaroni method (8) have shown that 31% absorption is preferable to the 30% used in the disc method, and the former level was therefore used in this study. Since results can be reproduced within 2 or 3%, the disc method is ideally suited to a kinetic study. In addition, it has been shown (1) that, with this method, pigment losses occur almost entirely during mixing.

The dried discs were ground in a semi-micro Wiley Mill to pass a 60-mesh sieve. Pigment was extracted with water-saturated butyl alcohol and determined in an Evelyn Colorimeter. All the pigment determinations were corrected to a 14% moisture basis.

### Results and Discussion

Preliminary experiments were made with a number of good and poor varieties to gain some knowledge of the characteristics of the reaction. Mixing was carried out at 30°C. in an atmosphere of air for intervals up to 10 mins. Typical curves for an excellent and a very poor variety are shown in Fig. 1. Other varieties were found to yield a similar type of curve with rates falling somewhere between these two. Fig. 1 indicates that the reaction occurs in three distinct

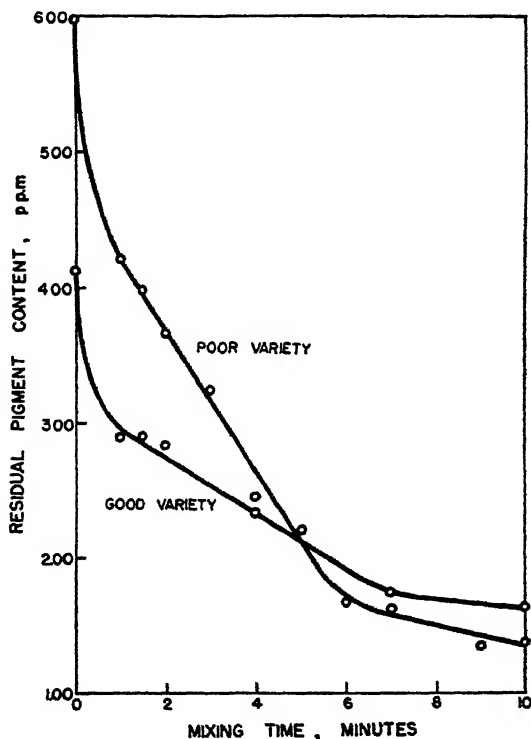


Fig. 1. Typical reaction curves for pigment loss during mixing of doughs representing a good and a poor variety of durum wheat.

stages: (1) a rapid reaction during the first minute of mixing; (2) a slower zero order reaction during the period from 1 to about 6 mins. of mixing; and (3) a still slower reaction, apparently zero order, beyond six minutes of mixing. The discussion in this paper deals only with the first two stages as it is unlikely that the third stage is reached during commercial processing of macaroni.

For convenience, the first two stages of the reaction will be called the "initial reaction" and the "mixing reaction" respectively. A distinction seems logical because the condition of the dough changes

after about 1 min. of mixing. At first, the revolving pins merely tumble the lumps of wet semolina through the fixed pins of the mixing trough. After about 1 min., a cohesive dough forms rapidly and the mixer does work on the dough through the shearing action that occurs as the revolving pins force the cohesive dough between the fixed pins.

Fig. 1 shows that the rates of both the initial reaction and the mixing reaction are much greater for the poor variety than for the good variety.

*Initial Reaction.* It is postulated that the initial reaction occurs merely as a result of wetting the semolina. This hypothesis was substantiated by the following experiment. Semolina cooled to  $-20^{\circ}\text{F}$ . was mixed with finely-ground ice at  $-20^{\circ}\text{F}$ . in an amount equivalent to 31% absorption. The mixture was then placed in a water bath at  $30^{\circ}\text{C}$ . for 1 hr.; as the ice melted, the water diffused evenly through the semolina and formed a dough, made *without mixing*, which could be handled in the usual way. The pigment lost by this sample (0.65 p.p.m.) was essentially the same as that lost by a control sample (0.62 p.p.m.) during the first minute of mixing, that is, during the initial reaction that occurs while the dough is merely tumbled.

It seemed possible that the initial reaction might be merely an artifact representing the difference between two experimental values; the first obtained by extracting the pigment from the dried semolina, and the second (and all subsequent values) obtained by extracting the processed discs. Any pigment destroyed during processing stages subsequent to mixing might thus appear as pigment destroyed by the initial reaction. In other words, when all experimental values other than the first are based on identical treatment of samples except for variations in mixing, any loss of pigment other than that occurring during mixing would probably be the same for all processed discs; as a result, the reaction curve would be lowered by a constant amount, and this might be interpreted to indicate an initial reaction which would then be an artifact rather than a reality.

To investigate this possibility, a sample was mixed and immediately extracted in the wet with a Waring Blendor. The pigment lost by this sample (2.20 p.p.m.) was essentially the same as that lost (2.23 p.p.m.) when a corresponding sample was processed into discs and dried. It was thus shown that the initial reaction actually does take place in the interval from zero time to 1 min. of mixing. Further support for this hypothesis was obtained in experiments on inhibition described in a later subsection.

*Mixing Reaction.* The foregoing experiment emphasizes a very interesting characteristic of the mixing reaction. Destruction of

pigment obviously occurs during mixing. When mixing is stopped, the destruction of pigment ceases even though the dough is handled wet through several subsequent stages and is finally dried over a period of 48 hrs. The explanation appears to depend on the emulsifying action of the mixer. The pigment, being fat soluble, is probably associated in the cell with the lipid fraction. After the addition of water and the formation of a cohesive dough, the lipid and aqueous phases are apparently emulsified by mixing thus enabling the reaction to proceed. It is also suggested that when the two phases are combined, the rate of the reaction is rapid by comparison with the rate at which the mixing reaction brings them together.

It has been assumed that the destruction of pigment during mixing is the result of an oxidation reaction catalysed by lipoxidase. Much

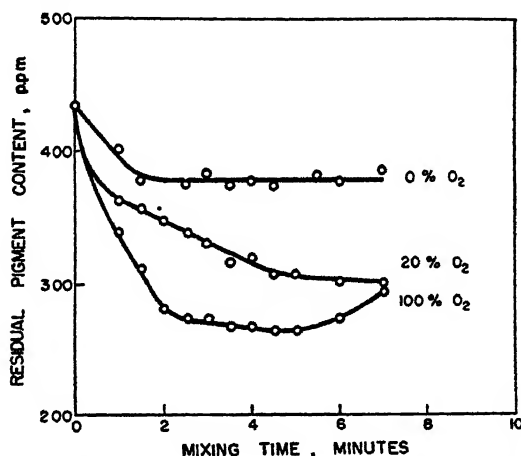


Fig. 2. Effect of mixing time and oxygen concentration on the loss of pigment from macaroni doughs.

work has been published in recent years on the coupled oxidation of carotenoid pigments during the peroxidation of unsaturated fats by lipoxidase. This work has recently been reviewed by Bergström and Holman (3). Moreover, lipoxidase activity has recently been reported in wheat flour by Miller and Kummerow (9).

*Oxygen Concentration.* Several experiments were made to determine the effect of oxygen concentration on the reaction. Samples were mixed at 30°C. in atmospheres of nitrogen, air, or oxygen. The results of one such experiment are shown in Fig. 2. The most interesting feature of this experiment is the course of the reaction under an atmosphere of nitrogen (curve labeled, 0% O<sub>2</sub>). The "initial reaction" takes place, but no further reaction occurs. Thus the mixing action produces no destruction of pigment in the absence of an atmosphere



containing oxygen. From a comparison of the reaction in air and in oxygen it is possible that the rates of both the initial and the mixing reactions increase with increasing oxygen concentration.

This experiment suggested that the initial reaction might be due to oxygen adsorbed on the semolina particles. Three samples were evacuated for three minutes and then nitrogen, air, and oxygen, respectively, were introduced. Each sample was then mixed under an atmosphere of nitrogen for three minutes to isolate the initial reaction. It was assumed that the evacuation would remove any adsorbed gases and that the nitrogen, air, or oxygen would subsequently be adsorbed on the semolina particles. No difference was found as a result of the

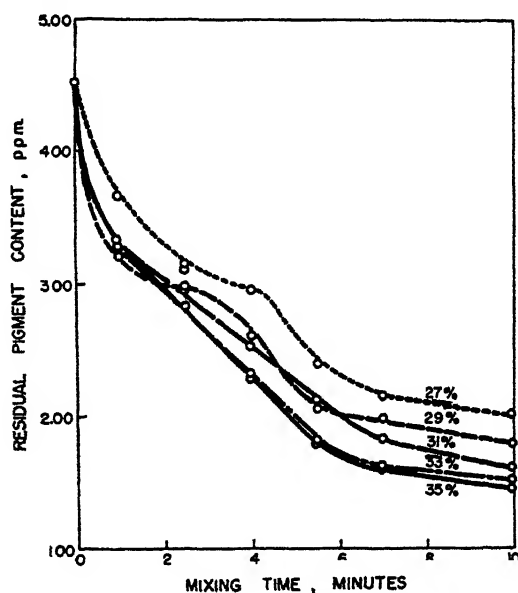


Fig. 3. Effect of mixing time and absorption on the loss of pigment from macaroni doughs.

possible adsorption of these different gases; the results were the same as that obtained with the original sample mixed for three minutes in nitrogen. Thus the initial reaction is apparently not due to adsorbed oxygen and must result from a more strongly held oxygen complex in the semolina—possibly within the unruptured cells of the original semolina particles.

**Absorption.** The effect of absorption was studied at five levels: 27%, 29%, 31%, 33%, and 35%. The results of the experiment are shown in Fig. 3. In general, the rate of the initial reaction is slower at lower absorptions, and there appears to be an induction period before the mixing reaction commences. The latter effect at low

absorptions probably results from slower formation of the cohesive dough that has been postulated as a necessary condition for the mixing reaction to proceed. There appears to be a limiting value for the absorption beyond which no increase in rate is apparent. This value was 33% for the particular sample used, but may vary from sample to sample as it is probably connected with the quantity and quality of the protein present.

*Temperature.* The effect of temperature on the reaction in air and in oxygen is shown in Fig. 4. It is evident that the temperature effect

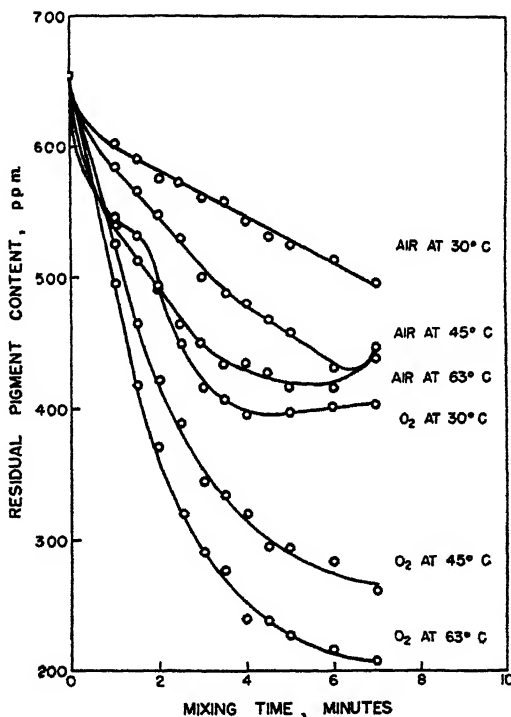


Fig. 4. Effect of mixing time and temperature on the loss of pigment from macaroni doughs.

is complex. The rate of the initial reaction increases with temperature in both air and oxygen and the rate of the mixing reaction increases as well. Because of the interaction of many factors, an interpretation of these results is difficult. Increasing temperature will hasten the formation of a cohesive dough. It will also affect the state of the dough and the rate of emulsification of the enzyme and lipid phases. In addition, it will displace any chemical equilibria involved in the reaction. It is interesting to note that the rate of the mixing reaction

is constant during some period at each temperature in air, while this is not true of any of the doughs mixed in oxygen. It is possible that when mixing is done in air, the partial pressure of oxygen is a limiting factor.

*Mixing in Nitrogen and Oxygen.* In the course of some auxiliary experiments on the effect of oxygen at higher temperatures, an interesting effect was observed which seemed to have a direct bearing on the mechanics of the mixing reaction. It was found that if mixing were carried on for some time under an atmosphere of nitrogen, and then this atmosphere was replaced by oxygen and mixing recommenced, a rather unexpected phenomenon occurred. Some of the experiments undertaken during this phase of the investigation are described below.

A sample was mixed at 30°C. under an atmosphere of nitrogen for 4 mins. Mixing was stopped and the atmosphere was replaced with oxygen. Mixing was then continued for 2 mins. The total mixing time was thus 6 mins. The loss of pigment in this sample was compared with the loss that occurred in a control sample mixed for 6 mins. in oxygen. The order of mixing was then reversed and a sample was mixed for 1½ mins. in oxygen followed by 3½ mins. in nitrogen. The loss in this sample was compared with that in a control sample mixed for 1½ mins. in oxygen only. The results were:

| <i>Mixing treatment</i>                               | <i>Pigment loss</i> |
|-------------------------------------------------------|---------------------|
| 1. 6 min. in O <sub>2</sub> at 30°C.                  | 2.52 p.p.m.         |
| 4 min. in N <sub>2</sub> + 2 min. in O <sub>2</sub>   | 2.77 p.p.m.         |
| 2. 1½ min. in O <sub>2</sub> at 30°C.                 | 1.20 p.p.m.         |
| 1½ min. in O <sub>2</sub> + 3½ min. in N <sub>2</sub> | 1.18 p.p.m.         |

This experiment provides striking verification of the emulsification hypothesis suggested in earlier discussions of the mixing reaction. It was proposed that the mixing action brought together the lipid and aqueous phases so that reaction could occur. It has been shown earlier that the mixing reaction does not take place when mixing is carried out under nitrogen. Yet a sample mixed for 4 mins. in nitrogen, then 2 mins. in oxygen, loses a similar amount of pigment to that of a sample mixed for the whole 6 mins. in oxygen. Hence it may be concluded that the two phases are brought in contact during mixing, but that no reaction can occur beyond the initial reaction until oxygen is introduced; once oxygen is introduced and mixing recommenced, the potential reaction which has been built up in the system by the mixing action can occur rapidly.

The second part of this experiment shows that the means by which oxygen comes into contact with the system is crucial to the progress of the reaction. If the dough is mixed for a short time in oxygen after a longer mixing period in nitrogen, the amount of pigment destroyed

depends on the oxygen concentration and on the *total* mixing time; but, if mixing is first done in oxygen, and nitrogen is then introduced, the reaction is immediately stopped. The partial pressure of the residual oxygen in the dough under an atmosphere of nitrogen will be small, and it thus appears that a certain minimum partial pressure of oxygen is necessary to bring about the pigment oxidation. This is one of the essential steps of the reaction.

Information on the rate of reaction in an atmosphere of oxygen after a preliminary treatment of 4 mins. mixing in nitrogen was sought by using a shorter mixing time in oxygen than that of the previous experiment. A control was run 4 mins. only in nitrogen. The following pigment losses were obtained:

| <i>Mixing treatment</i>                          | <i>Pigment loss</i> |
|--------------------------------------------------|---------------------|
| 1. Control, 4 min. N <sub>2</sub>                | 0.80 p.p.m.         |
| 2. 4 min. N <sub>2</sub> + 1 min. O <sub>2</sub> | 2.56 p.p.m.         |
| 3. 4 min. N <sub>2</sub> + 2 min. O <sub>2</sub> | 2.77 p.p.m.         |

These results indicate that the maximum rate of the mixing reaction under these conditions is at least 2.56 p.p.m. per minute.

The pronounced effect of oxygen concentration on this "delayed" mixing reaction was illustrated with a sample treated in a similar manner to the above except that air was used instead of oxygen:

| <i>Mixing treatment</i>                                | <i>Pigment loss</i> |
|--------------------------------------------------------|---------------------|
| 1. 4 min. in N <sub>2</sub>                            | 0.80 p.p.m.         |
| 2. 4 min. in N <sub>2</sub> + 1 min. in air            | 1.21 p.p.m.         |
| 3. 4 min. in N <sub>2</sub> + 1 min. in O <sub>2</sub> | 2.56 p.p.m.         |

The net loss due to the *delayed* mixing is then: in air,  $1.21 - 0.80 = 0.41$  p.p.m.; in O<sub>2</sub>,  $2.56 - 0.80 = 1.76$  p.p.m. Thus the rate in oxygen is approximately  $4\frac{1}{2}$  times the rate in air; that is, the rate is roughly proportional to the oxygen concentration.

*Inhibiting Agents.* The effects of the following enzyme inhibiting agents were examined: cyanide ion, mercurous ion, sodium chloride, sodium fluoride, copper sulfate, lead acetate, alpha naphthol, and ethyl alcohol. The concentrations used were either hundredth or thousandth normal except for ethyl alcohol, which was employed in 30% concentration. Of these reagents, cyanide, mercurous ion, copper sulfate, alpha naphthol, and 30% alcohol had a significant effect on the reaction. Mercurous ion produced a marked accelerating effect (negative inhibition) on both stages of the reaction. Copper sulfate accelerated the initial reaction only. Cyanide inhibited both the initial and the mixing reaction, and a ten-fold increase in the cyanide concentration had little further effect on the amount of inhibition. Alpha naphthol inhibited the initial reaction very markedly, but had less effect on the mixing reaction; while 30% alcohol accelerated the initial reaction and almost completely inhibited the mixing reaction.

The alcohol inhibition was examined over a range of alcohol concentrations, from 5% to 40%, at two representative mixing times. The results are shown in Fig. 5. The acceleration of the initial reaction is constant for all concentrations from 5% to 35%, and at 40% marked inhibition of the initial reaction is evident. Increasing concentrations from 10% to 35% inhibit the rate of the mixing reaction, the rate being zero at 35% alcohol. At 40% alcohol there is a marked reversal of the reaction during mixing, which requires further investigation.

The alpha naphthol inhibition appears to be an antioxidant effect rather than true inhibition; for the initial reaction and a portion of the mixing reaction are completely inhibited, and the oxidation of pigment then proceeds again during a later phase of the mixing reaction. The alpha naphthol probably competes successfully with the pigment as

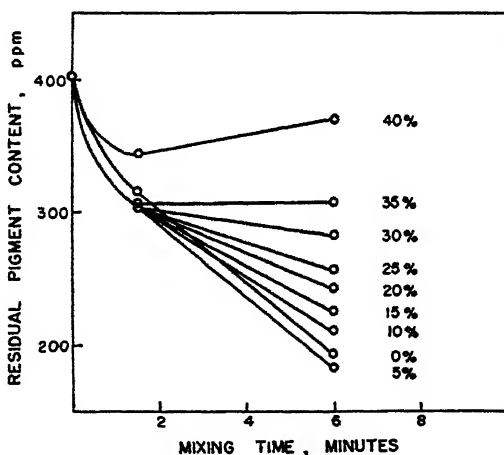


Fig. 5 Effect of mixing time and alcohol concentration on the loss of pigment from macaroni doughs.

substrate for the enzyme until it has been used up, after which the pigment oxidation again proceeds.

**Addition of Lipoxidase.** Probably the most convincing evidence that the enzyme lipoxidase is involved in the destruction of pigment during mixing is furnished by the behavior of the system on the inclusion of lipoxidase in the dough. The use of crude lipoxidase concentrates from soya bean meal for bleaching flour during the mixing of bread doughs has been common for over fifteen years. A sample of this concentrate bearing the trade name "Wytase" was obtained, and the amount recommended for bleaching bread flour doughs was added to several types of semolina before mixing. Two good varieties and one poor one were processed with this addition of enzyme. Added to the poor variety, the reaction rate was considerably increased but the

shape of the curve was the same as originally observed. The most striking effect was obtained with a sample of Mindum. The addition of "Wytase" produced a curve of the same type as that obtained normally with Golden Ball, a very poor variety. The control and "Wytase" curves are shown in Fig. 6 along with representative curves obtained for Mindum and Golden Ball. The data for the latter two samples are plotted, for ease of comparison, beginning at the same initial concentration; these values were actually 6.54 p.p.m. for Mindum, and 5.56 p.p.m. for Golden Ball. Thus it is possible, by the addition of a crude lipoxidase concentrate, to obtain the characteristics

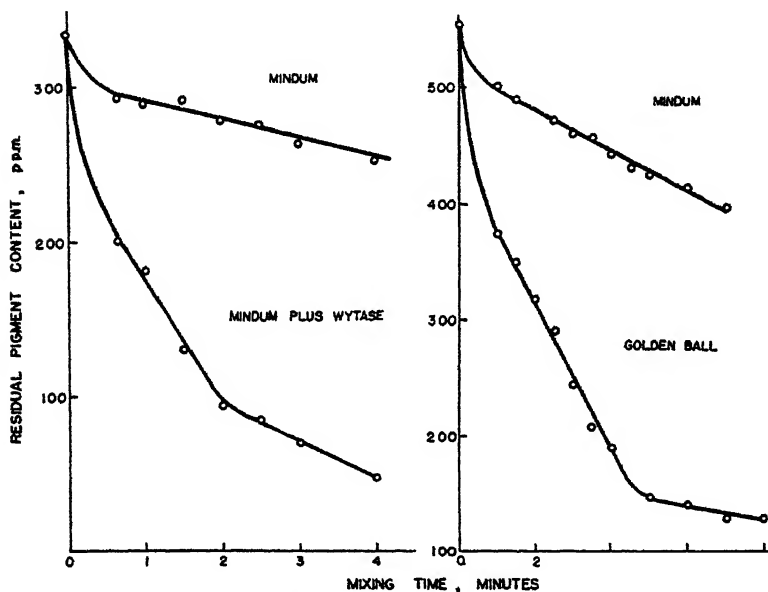


Fig. 6. Effect of added lipoxidase ("Wytase") on the loss of pigment for a good variety.

of a poor variety from a good variety and to make a poor variety considerably worse.

### General Discussion

Destruction of the carotenoid pigments during mixing involves an oxidation reaction. This is evident from the pronounced effect of oxygen concentration on the mixing reaction, and from the complete inhibition of the initial reaction and part of the mixing reaction by alpha naphthol, an antioxidant. It seems extremely probable that the products of the oxidation are no longer carotenoid-like, as it has been shown (7) that macaroni contains the same pigments as semolina, but in smaller amounts. Thus the oxidation products appear to contribute nothing to the yellow color of the macaroni.

It is thought that the oxidation involves an enzymatic reaction. Oxidation of carotenoid pigments by atmospheric oxygen is normally a slow process (4). The action of peroxides such as exist in ether solutions of pigments is likewise slow, occurring over a period of weeks. In the reaction under investigation, the oxidation occurs rapidly in a few minutes, and the presence of an enzyme thus seems highly probable. The ability of soybean lipoxidase to oxidize carotenoid pigments during its peroxidation of unsaturated fats is well established, and the presence of this enzyme in wheat germ has been demonstrated by Sumner (11) and in commercial flour by Miller and Kummerow (9). Calculation shows that the amount of lipoxidase in flour is more than sufficient to account for the rate of pigment destruction observed during mixing.

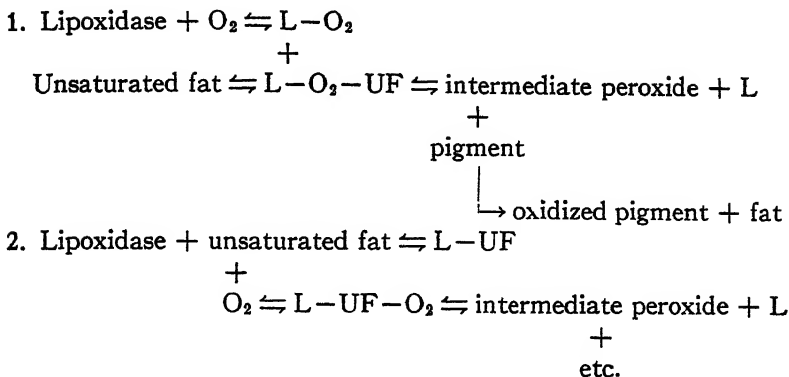
There are several other features of the reaction which point to an enzymatic origin. The effects of 30% alcohol in partially inhibiting the mixing reaction, and of 40% in totally inhibiting it, are suggestive; alcohol in these concentrations is known to denature protein, and would thus act as an enzyme inactivating agent. The effects of low concentrations of heavy metal ions and cyanide ion, the general shape of the reaction curve, and the low temperature coefficient of the reaction, are also characteristic of enzyme catalysed reactions. Finally, the effect of added lipoxidase on the reaction curve is perhaps the most convincing evidence for the enzymatic nature of the reaction. Samples which show little "enzymatic activity" behave in a manner exactly analogous to samples with high "enzymatic activity" when a small amount of "Wytase" is added to the semolina prior to mixing.

It has been established (6) that pure lipoxidase is not inhibited by cyanide ion. But early investigators (cf. 3), working with crude extracts, found inhibition by .001 *N* cyanide of the same order as was observed in these experiments. Thus it appears probable that *in vivo* the enzyme functions in conjunction with an activating system that is sensitive to cyanide. This suggestion is further justified by the fact that a ten-fold increase in cyanide concentration had very little additional inhibiting effect. It is suggested, on the basis of evidence noted earlier, that the activating system is accelerated by mercurous ion and possibly by the cupric ion as well.

It is generally accepted that alpha naphthol does not act as a true inhibitor of the lipoxidase system, but rather as an antioxidant. This appears to be its function in the system under investigation.

If we accept the hypothesis that pigment destruction is brought about by a coupled reaction involving peroxidation of unsaturated fat by lipoxidase, it appears that there are two alternate paths for the reaction to follow: either the lipoxidase combines first with oxygen or

first with the unsaturated fat. The two reactions may be written as follows:



The initial reaction appears to follow mechanism No. 1. It proceeds when mixing is carried out under nitrogen and also when the semolina is wetted but not mixed. Accordingly, it is postulated that the first step of mechanism No. 1, combination of lipoxidase and oxygen, has already occurred in the unwetted semolina in which the original cell structure of the wheat is still largely intact. It is also postulated that, at this stage, oxygen can diffuse into the cell but that lipoxidase and unsaturated fat are kept apart in immiscible phases; accordingly, the first step of mechanism No. 1 can take place but not the first step of mechanism No. 2. When water is added to the semolina it is rapidly imbibed and, according to the data of Baker, Parker, and Mize (2), the cells must rupture almost instantaneously. The subsequent steps of mechanism No. 1 can then occur. But the reaction soon ceases if either oxygen or mixing is withheld because the lipoxidase is not reactivated; for reactivation requires recombination of lipoxidase with substrate and oxygen which is thought to occur only as mixing exposes new surfaces of the dough to oxygen. Thus the initial reaction appears to be mainly a stoichichemical one that is limited to the amount of lipoxidase-oxygen complex formed in the semolina before water is added.

As an alternative hypothesis, it may be postulated that the initial reaction involves prior formation of peroxides in the semolina, and that these react with the pigment when water is added. This explanation is not considered tenable; for it has been shown (10) that peroxides cannot oxidize the pigment directly, but that this is accomplished by some transient intermediate formed during peroxidation of fats by lipoxidase.

There is evidence that the mixing reaction can follow mechanism No. 2 rather than mechanism No. 1. When dough is mixed under nitrogen



and then under oxygen, pigment destruction is the same as if all the mixing had been done in oxygen. Thus, during the mixing reaction, mechanism No. 2 must be operative; for it is evident that, while no oxygen is being supplied, a complex is building up in the system during mixing which needs only oxygen for rapid completion of the reaction. This rapid step occurs when oxygen is supplied and given ready access to the dough by continued mixing. Since this reaction is so rapid it seems likely that mechanism No. 2 is the preferred reaction; if conditions are suitable, it will occur rather than No. 1. But if, as in semolina during storage, the first step of mechanism No. 2 is blocked, while the first step of No. 1 is not, then the initial reaction will still take place by mechanism No. 1.

The integrated hypotheses outlined in the preceding paragraphs account for the principal observations made during the course of the investigation. Since the fundamental postulate, that the reaction is catalysed by lipoxidase, was developed as a result of early experiments, it was partially verified by the final experiment: the effects of adding lipoxidase ("Wytase") were predicted and subsequently confirmed. Additional verification of this and other parts of the hypotheses must await the results of further study. Alternately, it is possible that alternate and simpler hypotheses, adequate to account for all the facts, may be developed.

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## SOME CHARACTERISTICS OF THE STARCHES OF THREE SOUTH AMERICAN SEEDS USED FOR FOOD<sup>1</sup>

M. J. WOLF, M. M. MACMASTERS, and C. E. RIST<sup>2</sup>

### ABSTRACT

Starch was prepared by laboratory processing from seeds of *Amaranthus leucosperma*, *Chenopodium quinoa*, and *C. paludicaule* which are closely related to tumbleweed (*A. graecizans* L.) and pigweed (*C. album* L.). These seeds were found to contain 62.8, 61.5, and 51.1% starch (d.b.), respectively. The starch in each case occurred as very small granules, approximately 1 to 3  $\mu$  in diameter. The starch from *A. leucosperma* colored reddish-brown with iodine-potassium iodide solution and sorbed only 2.5 mg. iodine per g., thus resembling the starches of waxy varieties of cereal grains. The starches from *C. quinoa* and *C. paludicaule* colored blue with iodine-potassium iodide solution and sorbed 45 and 50 mg. of iodine per g., respectively. The three starches gelatinized within the range 48°–72°C. Starch of *A. leucosperma* formed a "long" paste, *C. quinoa* starch a paste of the same "length" as that of corn starch, while *C. paludicaule* starch formed a thin, watery suspension. None of the pastes gelled on standing.

As part of a general study of the character of natural starches, the starches obtained from a number of native and foreign plant species have been examined. In the course of this work, seeds of *Amaranthus leucosperma*, *Chenopodium quinoa*, and *C. paludicaule* were processed for starch.

The characteristics of these starches may be of interest to cereal chemists since the seeds from which they were prepared are used in some areas of South America similarly to corn, wheat, and other cereals grown in the United States.

Seeds of *Amaranthus leucosperma* are popped, similarly to popcorn, for food use. This plant is related to the tumbleweed, *Amaranthus graecizans* L., which is native to the United States. *Chenopodium quinoa* seeds are used for making porridge and ground to flour for preparing bread and cakes. The seeds of *Chenopodium paludicaule* are ground into meal which is used for making porridge and also in mixture with wheat flour for baking. The latter two plants are related to the common Lamb's Quarters, *Chenopodium album* L. In the United States, seeds of some species of both *Amaranthus* and *Chenopodium*, including *C. album*, are collected by the Indians and made into meal for use in cakes or gruel (4, pp. 129, 140).

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It is well known that starches from different tissues, even of the same plant, may have different characteristics. Our common commercial starches come from the endosperm of cereal seeds, and from modified roots and stems of several other plants. The three starches reported here may be of particular interest because they are stored in the perisperm of the seed. Little is known about starch from this type of tissue.

### Results and Discussion

Like our common cereal grains, these South American seeds contain starch as a major constituent. The amount of starch present is of the same order as that in wheat, but is appreciably less than in corn (Table I). Unlike the cereal grains, however, in which the starch is stored in the cells of the endosperm, the storage tissue in these seeds is the perisperm. In mature cereal grains the perisperm as a distinct tissue is completely lacking or is present only in negligible amounts and is of no importance as a starch storage tissue.

TABLE I  
STARCH AND PROTEIN CONTENTS OF SEEDS AND SOME PROPERTIES  
OF THE STARCHES

| Seed                           |                             |                          | Starch <sup>1</sup>           |                               |                                        |                                  |
|--------------------------------|-----------------------------|--------------------------|-------------------------------|-------------------------------|----------------------------------------|----------------------------------|
| Botanical name                 | Starch content <sup>2</sup> | Protein content N × 6.25 | Nitrogen content <sup>3</sup> | Granule diameter <sup>4</sup> | Color with I <sub>2</sub> -KI solution | I-sorptive capacity <sup>5</sup> |
|                                | % (d.b.) <sup>6</sup>       | % (d.b.) <sup>6</sup>    | % (d.b.) <sup>7</sup>         | μ                             |                                        | mg./g.                           |
| <i>Amaranthus leucosperma</i>  | 62.8                        | 16.1                     | 0.13                          | 1-3.5                         | Reddish-brown                          | 2.5                              |
| <i>Chenopodium quinoa</i>      | 61.5                        | 15.3                     | .17                           | 1.5-3                         | Blue                                   | 45                               |
| <i>Chenopodium paludicaule</i> | 51.1                        | 16.9                     | .12                           | 1-3                           | Blue                                   | 50                               |
| Corn                           | 70.9                        | 9.9                      | .04                           | 1-23                          | Blue                                   | 54                               |
| Wheat                          | 64.4                        | 14.7 <sup>8</sup>        | .04                           | 2-40                          | Blue                                   | 50                               |

<sup>1</sup> Starch was separated from the seeds by the second method described by MacMasters and Hilbert (3); this is a laboratory wet-milling procedure using only distilled water.

<sup>2</sup> Determined polarimetrically in a calcium chloride extract of the dry, ground seed (2).

<sup>3</sup> Method of (1) with Winkler's modification (6).

<sup>4</sup> Determined microscopically with the aid of a filar-micrometer eyepiece.

<sup>5</sup> Method described in (7). The values given are calculated on a protein-free basis.

<sup>6</sup> Moisture content was determined by drying the ground seeds for 1 1/2 hours at 130 C. in a forced-draft oven.

<sup>7</sup> Moisture determined as in (7).

<sup>8</sup> N  $\times 5.7$ .

The protein content of the seeds is similar to that of wheat but is considerably higher than that of corn (Table I). The high residual nitrogen content of the *Amaranthus* and the two *Chenopodium* starches indicates difficulty in separating protein from the small starch granules by the method used.

With respect both to color reaction with iodine-potassium iodide and iodine-sorptive capacity, the two *Chenopodium* starches are like the

TABLE II  
COMPARISON OF SOME PHYSICAL CHARACTERISTICS OF AMARANTHUS AND  
CHENOPODIUM STARCHES WITH THOSE OF CORN AND WHEAT STARCHES

| Source of starch              | Gelatinization range, <sup>1</sup> °C. | Relative paste length <sup>2,7</sup>    | Gel formation <sup>2,4</sup> |
|-------------------------------|----------------------------------------|-----------------------------------------|------------------------------|
| <i>Amaranthus leucosperma</i> | 55-72                                  | Considerably longer than corn starch    | None                         |
| <i>Chenopodium quinoa</i>     | 48-62                                  | Short; about same length as corn starch | None                         |
| <i>Chenopodium paludicane</i> | 56-71                                  | Shorter than corn starch; watery        | None                         |
| Corn                          | 52-72 <sup>5</sup>                     | Short                                   | Firm                         |
| Wheat                         | 51-64 <sup>6</sup>                     | Short                                   | Soft                         |

<sup>1</sup> Temperatures at which gelatinization starts and at which it is complete, respectively. Determined by slowly heating the starch suspension in a water bath and withdrawing samples for microscopic observation at intervals. Because the small size of *Amaranthus* and *Chenopodium* starch granules precluded the use of conventional methods, gelatinization range was determined by noting changes in size, form, and transparency of the granules. These data for corn and wheat starches were determined by loss of birefringence and by staining with benzopurpurin.

<sup>2</sup> All starches were defatted.

<sup>3</sup> Estimated by heating 5% starch suspension over a boiling water bath until thickened, cooling to about 30°C., drawing up the paste with a glass rod, and noting the length of the strand formed.

<sup>4</sup> Noted after storing the pastes at about 8°C. for 24 hours.

<sup>5</sup> Average values for many commercial samples.

<sup>6</sup> Average values for many laboratory-processed samples.

common starches of commerce, while the *Amaranthus* starch is similar to the starches of the waxy varieties of cereal grains (Table I).

The starches gelatinized over approximately the same range as ordinary corn and wheat starches (Table II). *Quinoa* starch gelatinized over a somewhat lower temperature range than the other two starches. The waxy character of the *Amaranthus* starch is emphasized by the long paste which it forms in comparison to non-waxy corn starch. The failure of the waxy *Amaranthus* starch to gel may be anticipated; however, the two non-waxy *Chenopodium* starches also yielded no gel on standing. Small granule starches from other plants, such as dasheen and rice, have been observed also to form either weak gels or none at all.

More than a dozen plants are mentioned in Meyer's (5) summary of the sources of starches which are colored red in the presence of iodine solution. Waxy corn, waxy *Coix*, and waxy barley starches have been added to the list since that time. As far as we know, however, *Amaranthus leucosperma* is the first plant of commercial importance, other than the cereal grains, which has been found to contain this type of starch.

#### Acknowledgment

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## KERNEL HARDNESS IN CORN. I. A MACHINE FOR THE RAPID DETERMINATION OF KERNEL HARDNESS<sup>1</sup>

EDMOND H. BENNETT<sup>2</sup>

### ABSTRACT

An electrically operated machine has been devised for the rapid determination of hardness of grain. A mechanical feeder delivers grain at a uniform rate between an inner driven wheel rotated at 33 r.p.m., and an outer wheel which rotates only when grain is being crushed, since it is propelled by the pinning action of the crushing grain.

Indexes to hardness are obtained by a hydraulic piston-regulated, recorder-unit which is driven by the outer crusher wheel when it rotates. The hydraulic pressure is generated in an hydraulic cylinder, the plunger of which is actuated by the torque transmitted to the crusher frame by the crusher wheels. Either the number on the recorder or the hydraulic pressure, as registered on a pressure gauge, may be used as an index of hardness.

The coefficient of variability ranged between one and three per cent for tests on samples of corn.

For some years livestock feeders and many agricultural investigators have been interested in the determination of corn hardness to ascertain the relationship between hardness and feeding value. Moreover, the rapid determination of seed hardness should be of value, particularly to agronomists and millers, since hardness is related to certain physical and chemical properties.

Various methods have been used to arrive at indexes of corn hardness. Robison (4) compared the per cent of starchy kernels in hybrids and open-pollinated varieties of corn. The kernels were examined over a glass-topped box containing an electric light. If the opaque

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area extended the full length of the kernel it was termed starchy. Culbertson, Shearer, Hammond, and Robinson (2) measured the hardness of kernels on edge between the jaws of a machine applying pressure until the kernel was crushed. Veach (5) built an hydraulic seed hardness tester and used it on various kinds of seeds, including clover, Lespedeza, sweet clover, and corn. Bennett (1) used the Veach machine to study the influence of such variables as shape, size, moisture content, and storage time on the crushing strength of corn. All of the tests mentioned above were made on a number of individual

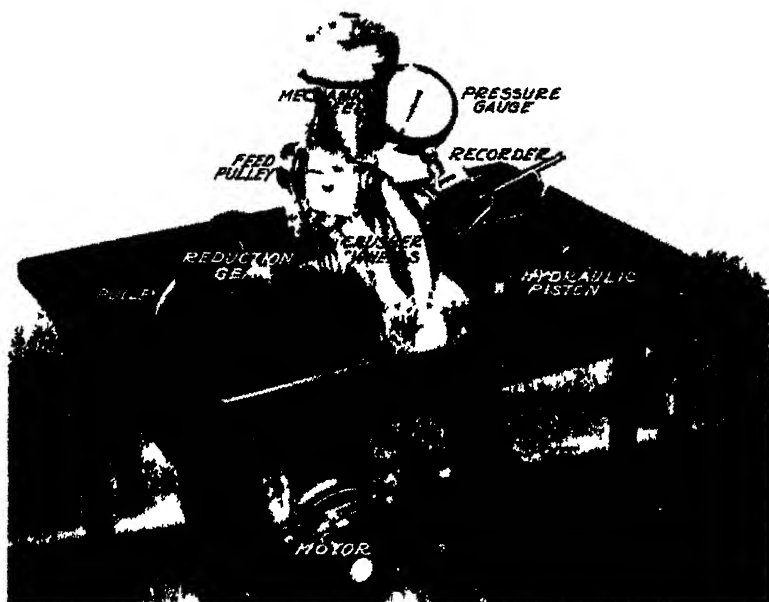


FIG 1 Left rear view of crusher. Details of mounting on steel and wood platform are shown. The legs on the platform make possible the removal of the crusher from its stand to a table or bench if desired. The stand has rollers which make it easily portable. The flexible crushed corn spout shown at lower right carries the corn away. The rear end of the frame is raised a variable distance in crushing, while the front lowers and forces a piston downward.

kernels. Bennett (1) also determined corn hardness by a method developed by Cutler and Brinson (3) for testing wheat. This method is based on the fact that a ground sample of hard seeds yields a higher per cent of large sized particles than softer seeds.

The early attempts to determine corn hardness by crushing a number of kernels individually and using the average crushing pressure proved slow and arduous. The extreme variability of hardness made it necessary to test large numbers of kernels to obtain means with low errors. Three machines were built successively in the attempt to perfect a simple, dependable method of obtaining quick, accurate, and

valid tests by utilizing *aggregate* samples. This paper describes the construction and operation of the third machine, the one currently in use.

### Materials and Methods

Fig. 1 is a photograph of the machine, left rear view, and Fig. 2, a photograph of a plan view. The principle of operation is illustrated by the schematic drawing, Figs. 3 and 5. All the metal parts of the machine are polished and cadmium plated to prevent corrosion.

*Automatic Feeder.* A sample of seed is placed in the hopper which is constructed of 16 gauge stainless steel and has a capacity of ap-

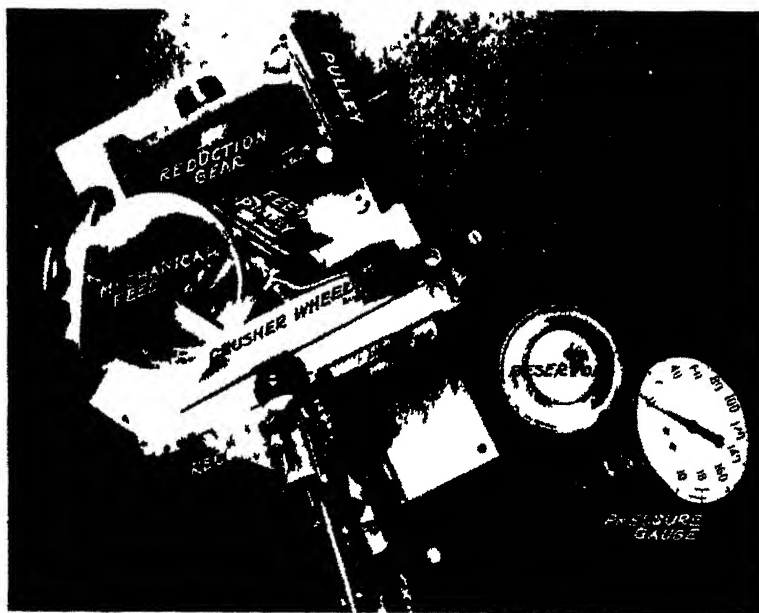


FIG 2 Plan view of crusher Through the open hopper may be seen the details of the mechanical feed Position of recorder on the outer crusher wheel is also shown Three kernels are shown in one of the roller groves

proximately 300 gms. of corn. The automatic feeder then delivers the seeds in a uniform stream to the crusher wheels. Because the seeds must be delivered to the crusher at a uniform rate, the automatic feeder is a very important part of the machine. Fig. 2 shows the diagonally grooved roller located at the bottom of the hopper. This roller feeds the seeds to the crusher. It is driven from the drive shaft of the crusher wheel by means of sheaves and a coiled spring belt. Four roller speeds are possible by the use of two steps on each sheave. Rate of feed may be further regulated or shut off entirely by means of a steel plate which slides into the bottom of the hopper.

*Crushing Mechanism.* The seeds are crushed between the outer surface of the inner wheel and the inner surface of the outer wheel. The position of the wheels (Fig. 4) is made possible by supporting each wheel independently in a rigid steel frame. The inner wheel (5 in. d.  $\times$   $\frac{9}{16}$  in. w.) is driven by, and is integral with a shaft (Fig. 4, No. 2). This shaft turns in a bronze bearing and is attached by a rigid coupling

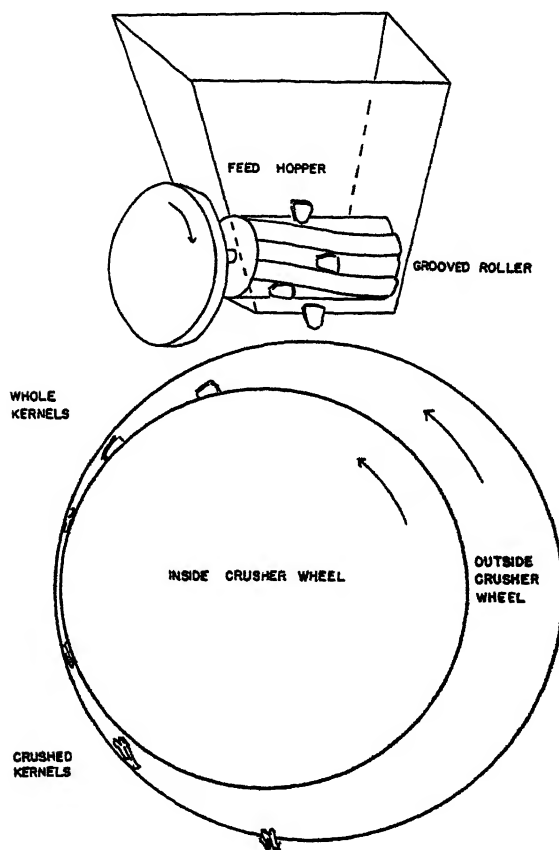


FIG 3 Schematic drawing of crusher, showing principle of operation

to the drive shaft of the speed-reduction gear. The outer crusher wheel (7 in. d.  $\times$   $\frac{9}{16}$  in. w.) is supported by an eccentric shaft (Fig. 4, No. 4) on the crusher frame, opposite the drive shaft of the inner wheel. The shaft surface is eccentric in relation to its support in the crusher frame so that the distance between the crushing surfaces can be regulated from 0 to  $\frac{1}{4}$  in. by turning the shaft in the frame. The



desired clearance is maintained by tightening the shaft retaining nut. The outer wheel turns freely on the outer bronze bearing surface of the shaft. The crusher frame has holes drilled to the bronze bearings, fitted with Alemite grease fittings for lubrication. The frame (Fig. 4, No. 5) is free to rock about its two concentric supports (Fig. 4, No. 6). These bearing supports are contained in pedestals welded to the base plate of the apparatus.

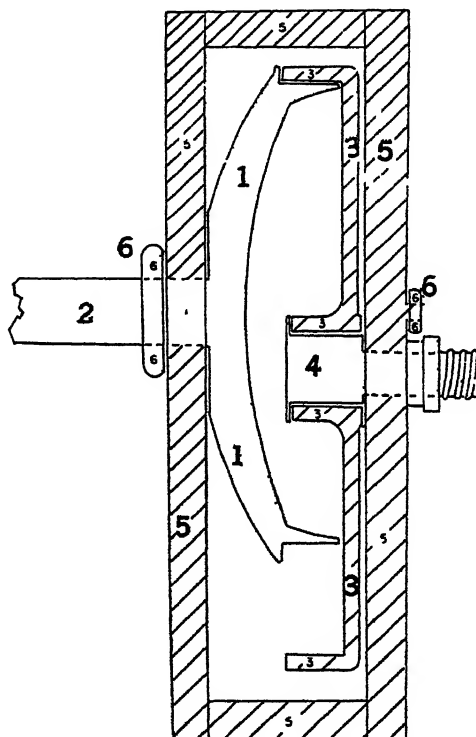


FIG. 4. Cross section, top view. 1 Inner crusher wheel integral with driving shaft No. 2. 2 Shaft which supports and drives inner crusher wheel. It is connected by a rigid coupling to the reduction gear. 3. Outer crusher wheel. 4 Stationary, offset shaft which supports outer crusher wheel. Turning this shaft adjusts crushing clearance. 5. Steel frame which supports crusher wheels, and rocks in frame supports No. 6. 6. Supports for frame.

*Principle of Force Transmission.* The resistance of the seeds to crushing produces separating forces acting upon the two crusher wheels. These forces will occur in the "crushing zone" (Fig. 5) and their resultant will be the equal and opposite forces "A" acting on the two wheels.

These forces are transmitted through the two crushing wheels to their respective bearings in the frame. The forces acting on the frame are labeled "B" in Fig. 5, and constitute a couple or torque equal to the

product BL which tends to rotate the frame (Fig. 4, No. 5) in its supports (Fig. 4, No. 6). "L" is the distance between the two lines of force "B" (Fig. 5).

The frame is restricted from rotating by means of hydraulic pressure on an hydraulic piston (Fig. 5). The hydraulic pressure (which is proportional to the frame torque and an index to seed hardness) is transmitted to a pressure gauge, and also to a mechanical integrating device which actuates a counter. The counter reading is proportional to the average hydraulic pressure, and therefore an index of the average hardness of the sample tested.

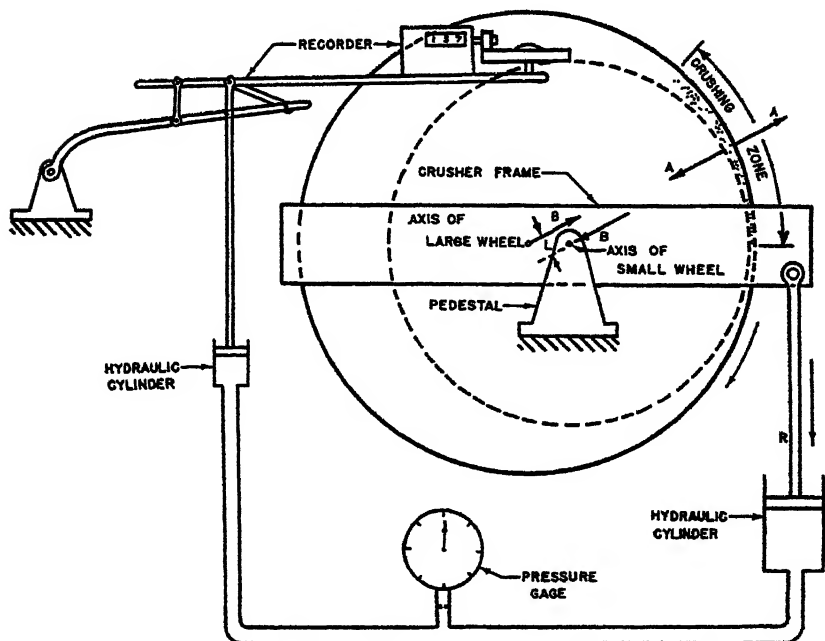


FIG. 5. Schematic drawing of crusher illustrating forces involved in crushing corn and in measuring the forces.

*Recording of Forces.* The recorder is composed of the integrating device and the counter mentioned above. The integrating device is actuated by hydraulic pressure, and the counter is turned by a rubber wheel which rolls on the side of the outer crusher wheel when seeds are being crushed. A rod (R) connects the crusher frame to an hydraulic piston. This piston produces an hydraulic pressure proportional to the torque developed, and is registered on a pressure gauge in units of 0.15 lbs. per sq. in. The pressure fluctuates during the testing of a sample. This is especially true with corn. The effect of these fluctuations on the indicator hand of the gauge is dampened by the metering effect of a

constriction produced by a small hole drilled through a block installed in the line of the gauge. Copper tubing transmits the hydraulic pressure also to a second cylinder where the force on a piston regulates the position of the recording device (modified r.p.m. counter) working on the outer side surface of the outer crusher wheel (Figs. 1, 2, and 5). The greater force required to crush hard seeds causes the recorder to operate near the periphery of the outer crusher wheel. The recorder wheel, therefore, revolves rapidly; and a relatively large number is registered on the recorder for a definite weight of seeds. Soft seeds, on the other hand, need less force and the recorder operates nearer the center of the crusher wheel. The recorder wheel, then, revolves more slowly and a smaller number is registered on the recorder. When the recorder number is used as an index to hardness it is necessary that the samples be weighed accurately. The indexes are objective and eliminate the human element to a large extent.

The other index of hardness, the hydraulic pressure as registered on the gauge, is a more sensitive measure than is the recorder number. Besides, this method eliminates the necessity of weighing the samples. However, since the pressure fluctuates during the testing of a sample, the accuracy of the estimate of hardness is dependent, to some extent, on the skill and judgment of the operator.

### Results

With the machine described above, approximately 3,000 hardness tests, mainly of 200 g. samples of corn have been made. The corn was grown at several stations and included systematic single crosses of midseason inbreds, inbreds, double cross hybrids, and special types of open-pollinated corn. The machine was also used for testing soybeans and wheat. It has a distinct time-saving advantage over methods where kernels are tested individually. When the recorder number was used as a hardness index, approximately forty to fifty samples per hour were weighed and tested by an experienced operator. When the hydraulic pressure was used directly as a hardness index, testing was more than twice as fast.

*Validity.* The results agreed with the physical appearance believed to be associated with hardness in corn. Illinois "high protein" corn has small kernels which appear very dense and have little or no floury endosperm. When tested it proved considerably harder than Illinois "low protein" which has larger kernels with the endosperms almost entirely floury (Table 1). Inbred 38-11 which was considered to contribute hardness to hybrids, proved much harder than inbred Indiana 66, which was considered to contribute softness to hybrids. A hard and a soft hybrid corn was tested for hardness on

both the above described machine and the Veach (5) machine, mentioned earlier, to compare their results with the physical appearance of the corn. The two hybrids were bred especially for testing purposes. One (38-11  $\times$  5677) (307  $\times$  Kys), was grown from inbreds selected for their ability to contribute hardness to the hybrids in which they were used. The inbreds which made up (WF9  $\times$  CC1) (Ind. 66  $\times$  Hy) were believed to contribute relative softness to hybrids. The physical appearance of the two hybrids is illustrated by Fig. 6. When they were tested for hardness with the machine described in this paper, the softer appearing hybrid had an index of 56, on the recorder, and the harder hybrid had an index of 72. However, when individual kernels of these same hybrids were tested on the Veach machine, the softer

TABLE I  
HARDNESS TESTS ON SAMPLES OF GRAIN FROM CORN GROWN AT ILLINOIS  
AGRICULTURAL EXPERIMENT STATION, 1944, EACH TEST 200 GRAMS

| Corn                                     | Mean<br>Recorder<br>Score               | Pressure<br>Readings |
|------------------------------------------|-----------------------------------------|----------------------|
| (38-11 $\times$ 5677) (307 $\times$ Kys) | 72 <sup>1</sup> $\pm$ 0.73 <sup>2</sup> | 92 <sup>3</sup>      |
| (WF9 $\times$ CC1) (Ind. 66 $\times$ Hy) | 56 $\pm$ 0.41                           | 58                   |
| III. "high protein"                      | 60 $\pm$ 0.55                           | 65                   |
| III. "low protein"                       | 52 $\pm$ 0.34 <sup>2</sup>              | 48                   |
| Inbred 38-11                             | 62 $\pm$ 0.70                           | 70                   |
| Inbred Ind. 66                           | 51 $\pm$ 0.80                           | 44                   |
| hh (defective endo.)                     | 52 <sup>4</sup>                         | 40                   |
| fl <sub>2</sub>                          | 48 <sup>4</sup>                         | 35                   |

<sup>1</sup> The recorder score represents, in effect, the product of an average crushing pressure and duration of test.

<sup>2</sup> Standard error of mean recorder score of three to seven tests, the number depending on the amount of corn available for testing.

<sup>3</sup> While testing, the operator estimates the average pressure from the gauge, expressed in units of 0.15 p.s.i.

<sup>4</sup> Sufficient corn for only two tests was available from these samples.

appearing hybrid (WF9  $\times$  CC1) (Ind. 66  $\times$  Hy) tested considerably harder than the other. An examination showed that a smaller surface area of the rounded kernels of the harder hybrid (38-11  $\times$  5677) (307  $\times$  Kys) touched the flat, parallel jaws of the machine than was the case with the softer hybrid. It is thought that the smaller contact area caused a localization of stress which allowed the more rounded kernels to be more easily crushed even though they were harder than the flat ones of (WF9  $\times$  CC1) (Ind. 66  $\times$  Hy). Results indicate that the rounded crushing surfaces of the machine described in this paper tend to minimize the effect of kernel shape on crushing resistance.

*Variability of Individual Kernel Tests Compared With Aggregate Tests.* The most striking observation made while testing hardness by

crushing individual corn kernels with several individual kernel machines was the extreme difference in their hardness. When such tests were run using the Veach machine on the above mentioned hybrids, 120 kernels of (WF9  $\times$  CC1) (Ind. 66  $\times$  Hy), with a mean crushing resistance of 164.5 lbs. had a standard error for individual tests of  $\pm 97.0$  lbs. One hundred twenty kernels of (38-11  $\times$  5677) (307  $\times$  Kys), with a mean crushing resistance of 109.0 lbs. had a standard error for individual tests of  $\pm 86.0$  lbs. Similar tests were run on inbred 38-11 to determine whether inbreds varied as much as hybrids. One hundred kernels had a mean crushing resistance of 112

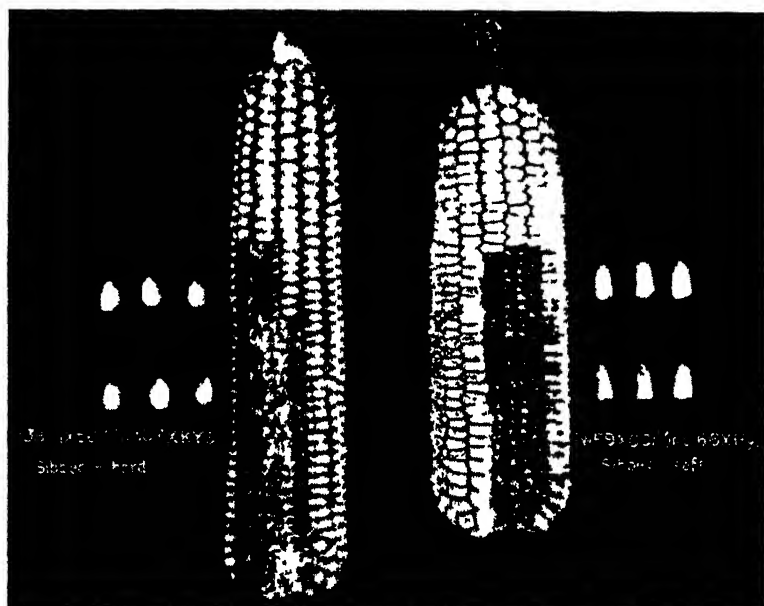


FIG 6 Two hybrids produced from inbreds selected for their hardness contributing qualities.

lbs. with a standard error of 56.0 lbs. It was found in testing Illinois Station Yellow Dent, very closely graded for size through both round and long holed screens, that it was necessary to test 273 kernels to obtain means that were significant at the 5% level, within the limits,  $\pm 10.0$  lbs.

Using the recorder score of the machine under consideration, hybrid (38-11  $\times$  5677) (307  $\times$  Kys) with a mean hardness index of 72.0, had a standard error of  $\pm 1.29$  for individual tests. Hybrid (WF9  $\times$  CC1) (Ind. 66  $\times$  Hy), with a mean hardness index of 56.0, had a standard error for individual tests of  $\pm 0.71$ . Although the units of measurement differ in the two types of tests, it is evident that the

machine described here, has materially reduced the error of hardness tests.

*Other Seeds.* When the crusher was used for testing soybeans no change was found necessary in the crusher clearance or the rate of feed. Soybeans proved softer than corn, and there was less fluctuation in crushing pressure. However, tests showed considerable difference between the varieties tested. Tests made on wheat without changing the settings from those used with corn were not satisfactory. Some of the wheat passed through without being crushed and the feeder delivered the wheat to the crusher too fast. With appropriate adjustments, however, it is possible that wheat also could be tested accurately.

#### Acknowledgment

The author expresses his thanks to G. H. Dungan, Professor of Crop Production at the University of Illinois, for advice concerning Agronomic problems. He also thanks Professors S. Konzo, R. C. Juvinall, and C. D. Greffe of the Department of Mechanical Engineering, for helpful advice concerning engineering terminology and description.

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## KERNEL HARDNESS IN CORN. II. A MICROSCOPIC EXAMINATION OF HARD AND SOFT TYPES OF DENT CORN<sup>1</sup>

EDMOND H. BENNETT<sup>2</sup>

### ABSTRACT

A close correlation was found between observed structure of the corn kernels and hardness as measured with the machine described in the foregoing article. Mature kernels of hard types of dent corn have smaller starch granules and more dense appearing protein matrix than softer types of dent corn. The amount of floury endosperm was found to be greater for the softer corns.

In wet milling, the aims are to remove all the starch from its matrix, and to remove all other cell contents from the starch. The more incomplete their separation, the poorer is the quality of the starch, or the lower is the per cent of starch return from corn. In the production of corn flakes, large grits are sought that are free from checks. Such grits, when properly softened, can be rolled out into large uniform flakes. Corns vary in the extent to which they satisfy the above demands, undoubtedly due to structural differences. Since starch and its proteinaceous matrix form most of the endosperm, their relationship is important in a study of its structure.

Cox, MacMasters, and Hilbert (3) observed that the smallest starch granules were found in the cells immediately beneath the aleurone layer where they are imbedded in a heavy protein matrix. They also noted that during processing most of the granules from this location contributed to tailings and relatively few to the prime quality section of starch.

It is the purpose of the present study to compare the structure of kernels which contrast widely in hardness, and point out the possible significance to the processing of corn.

### Materials and Methods

Samples of mature kernels from three groups of corn were tested for hardness employing the machine described by Bennett (1). The hardest and softest from each group, as well as four other corns were selected for microscopic examination of structural characteristics.

<sup>1</sup> Manuscript received August 12, 1949. From the Department of Agronomy, University of Illinois, Urbana, Illinois.

<sup>2</sup> Instructor in Biology, Division of General Studies, University of Illinois, Urbana, Illinois.

These included: a rice type popcorn, a Peruvian flour corn, "Cuzco";<sup>3</sup> two double cross hybrids, one bred for its hard and the other for its soft characteristics; one hard and one soft single cross hybrid; one hard and one soft inbred; Illinois "high protein" corn with a protein content of approximately 20%, relatively hard, and Illinois "low protein" corn, relatively soft, with a protein content of approximately 5 to 6%.

*Sectioning.* The mature corn kernels were soaked 28 hrs. in distilled water at 36°C. and sectioned with a sliding microtome using a carbon

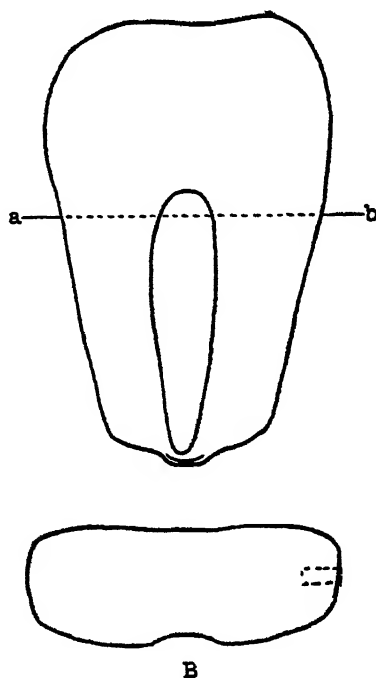


FIG. 1. A. Longitudinal outline of corn kernel, a-b represents point from which transverse sections were taken. B. Face view outline of a transection. Broken line rectangle indicates area photographed.

dioxide freezing attachment. Transverse sections of the corn kernels were made at the median of the longitudinal axis (see Fig. 1A). Photomicrographs were taken of a part of the sections from the aleurone layer inward about one-fourth the distance to the center (Fig. 1B) of the sections. The sections were approximately  $20\ \mu$  in thickness, with the exception of popcorn which was about  $12\ \mu$ , and Cuzco, which was about  $80\ \mu$ . The sections were stained with safranin and

<sup>3</sup> Popcorn and Cuzco were not tested for hardness but were examined microscopically because they represent, respectively, the extreme flinty type and the extreme floury type of endosperms.



haematoxylin and very lightly with iodine (ten p.p.m.). The staining procedure was regulated so that the assembled colors were: pericarp—bright red; aleurone layer—dark with some cells clear where the contents had dropped out; cell walls—dark violet to brown; protein matrix—light yellowish brown; embryo—violet; starch granules—very light blue; nuclei—dark violet. Some sections were stained as above but with iodine omitted from the procedure and the coloration of the nuclei was the same as those with the iodine included. The sections were mounted temporarily in water and photomicrographs made.

Starch granules from each of the corns studied, with the exception of the two single crosses and two inbreds, were examined under the microscope and measured with an ocular micrometer. The final measurement was calculated after checking with a stage micrometer. The kernels were first soaked in water, then sectioned with a sliding microtome at the location indicated in Fig. 1B. Strips were cut from three areas of the sections located as follows: area I was located immediately beneath the aleurone layer; area II, midway between the aleurone layer and the center of the kernel; and area III, in the center of the section. These small strips were placed on a microscope slide in a drop of water and the starch granules teased out. A drop of dilute iodine solution with a concentration of approximately ten parts per million was then added and the measurements made.

### Results

A cross section of a dry mature dent corn kernel shows the endosperm with two distinct regions, the horny endosperm and the floury endosperm. The floury endosperm is opaque, friable and easily crushed between the fingers. Upon microscopic examination, in contrast to the horny endosperm, it has thinner cell walls and less dense protein matrix, appearing incomplete in places, around the starch granules. The starch granules are more loosely arranged and more uniformly spherical in shape in the floury endosperm. The horny endosperm, itself, varies in structure. All the corns examined showed a general pattern as follows: one to several rows of cells just inside the aleurone layer are distinguished by their small size, dense protein matrix, small starch granules, and thick cell walls. Centripetally the cells, as well as the starch granules are progressively larger in size, and more variable in size and shape. The starch granules are progressively more crowded farther from the aleurone layer toward the inner limits of the horny endosperm. The closely packed granules become distorted in shape in contrast to the roughly spherical shape of the less crowded granules nearer the aleurone layer.

*Popcorn and Cuzco.* The present study confirmed structural differences found by Cox *et al.* (3) between popcorn and Cuzco. Starch granules were smaller and the protein matrix more dense in the horny endosperm of popcorn than for corresponding areas in the horny endosperm of Cuzco. These differences are illustrated by Fig. 2. The nuclei in the cells of popcorn endosperm are considerably larger.

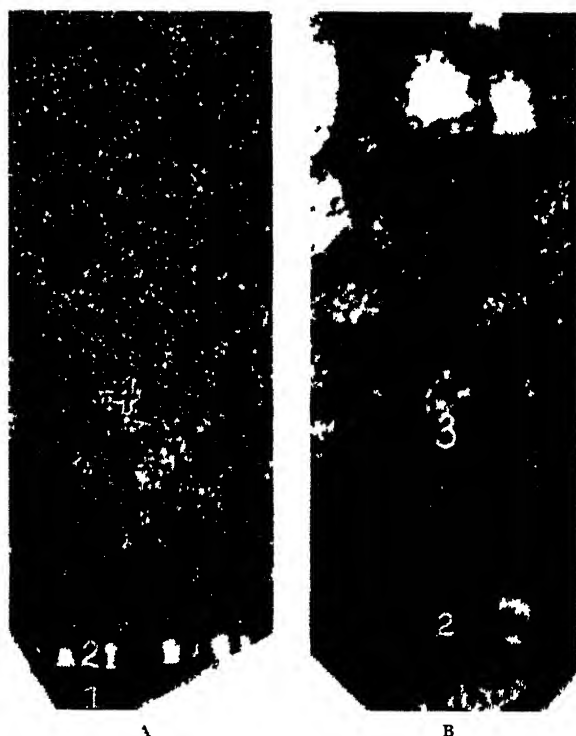


FIG. 2. A. Photomicrograph of cross section of popcorn showing cellular relationship 1. Pericarp; 2. Aleurone layer, 3. Cell of horny endosperm; 4. Nucleus.  $\times 100$ . Thickness  $12\ \mu$ . B. Cross section of Cuzco corn kernel. Pericarp (1) is shown at bottom. Aleurone layer (3) is two cells thick in places. Cells (3) are larger than those of popcorn. Starch granules are larger. Upper left corner of photomicrograph includes the edge of the floury endosperm.  $\times 100$ . Thickness approx  $80\ \mu$ .

*Dent Hybrids and Inbreds.* Fig. 3 illustrates the structural differences found between hard and soft dent corn. The hard double cross (38-11  $\times$  5677) (307  $\times$  Kys), the hard single cross (L317  $\times$  38-11), and the hard inbred (Ill.90) contained a greater proportion of horny endosperm than did the softer corns (WF9  $\times$  CC1) (Ind. 66  $\times$  Hy), (K155  $\times$  WF9) and CC1. For corresponding areas within the horny endosperms of the harder corns the protein matrix appeared more dense, the starch granules smaller and the nuclei more prominent than

in the softer corns. The nuclei of the hard inbred, Ill.90, were not as large as those of the two hard hybrids.

*Illinois "high protein" and Illinois "low protein."* The contrast in macroscopic appearance between sections of Illinois "high protein" kernels and sections of Illinois "low protein" kernels was almost as great as between sections of popcorn and Cuzco kernels. The kernels of "high protein" were the smaller and harder of the two. They con-

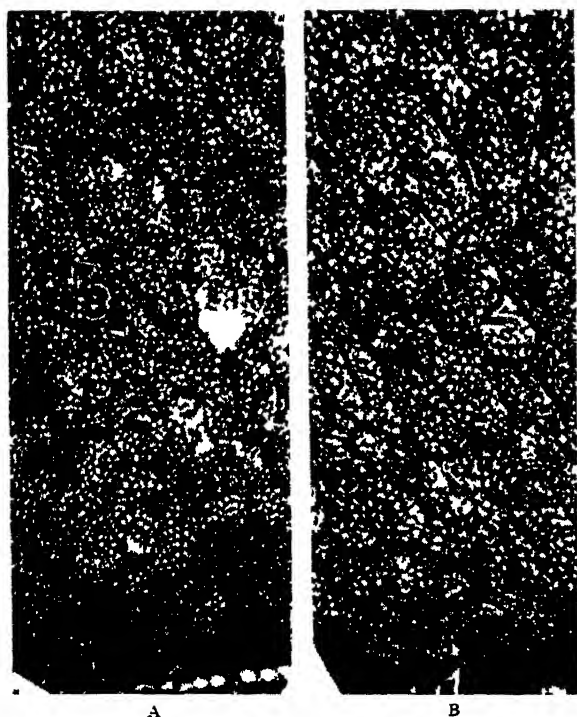


FIG. 3. A. Photomicrograph of cross section of hard hybrid (307  $\times$  K<sub>3</sub>s) (38-11  $\times$  5677) corn kernel. 1. Aleurone layer, 2 Endosperm cells, 3. Nuclei.  $\times$  100. Thickness approx. 20  $\mu$ . B. Cross section of soft hybrid (WF9  $\times$  CC1) (Ind. 66  $\times$  Hy). Aleurone cells (1), endosperm cells (2), and starch granules are larger than those of the harder hybrid. Nuclei are less conspicuous.  $\times$  100. Thickness approx. 20  $\mu$ .

tained a small amount of floury endosperm at the crown, but very little or none back of the scutellum. The floury endosperm of "low protein" kernels extended from the distal to the chalazal end and in some kernels the horny endosperm occurred only as a thin shell around the floury endosperm. Microscopic examination showed that "low protein" kernels possessed larger starch granules in areas I and II (Fig. 4). However, the starch granules from the center (area III) of "low protein" kernels were smaller than for area II, and the starch granules

from area III of "high protein" kernels were larger than those found anywhere else in the same or different kinds of corn that were examined

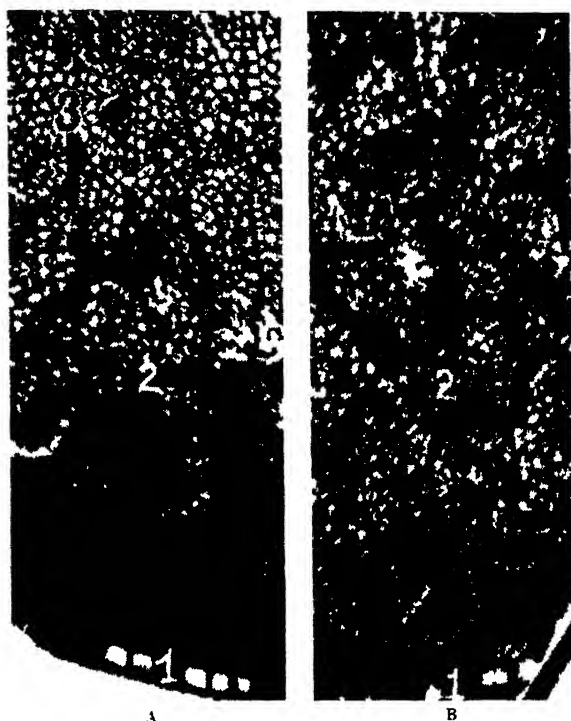


FIG. 4. A. Photomicrograph of cross section of Illinois High Protein kernel. 1. Aleurone cells, 2. Endosperm cells; 3. Prominent nuclei.  $\times 100$ . Thickness approx.  $18 \mu$ . B. Illinois low protein kernel. 1. Aleurone cells. 2. Endosperm cells. Starch granules are larger in the area shown above. Nuclei are less conspicuous.  $\times 100$ . Thickness approx.  $25 \mu$ .

in the present work. The protein matrix was more dense in the "high protein" corn, and the nuclei were especially large and conspicuous.

### Discussion

The results of the present work with dent corn indicate that hardness is related to certain structural characteristics. The harder corns examined had smaller starch granules in a denser appearing matrix than the softer corns. The work of Cox *et al.* (3) indicated that the smaller starch granules in a massive protein matrix were contributed to tailings. In softer corn with its larger starch granules the separation of starch should be more complete resulting in a higher per cent of starch return and a greater percentage of high quality starch. This expectation needs to be verified by further tests, however.

Although considerable effort is expended on methods of processing corn to produce better finished products, present methods of quantity buying discourage attempts to determine the characteristics that are desirable in the raw product. Since wet millers normally produce starch from low, or sample grade corn, it seems improbable that the present research will directly influence their choice of raw material. The main conclusion to be drawn from the work presented here is that the differences found by Cox *et al.* (3) between popcorn and flour corn also occur, in a less degree, between hard and soft varieties of dent corn. This may be useful as a partial explanation of differences observed by wet millers in steeping different lots of corn.

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## AMINO ACID COMPOSITIONS OF WHEAT AND CAROB GLUTENS<sup>1</sup>

A. C. RICE<sup>2</sup> and P. E. RAMSTAD<sup>3</sup>

### ABSTRACT

A material resembling wheat gluten was prepared from the germ flour of the carob seed, *Ceratonia siliqua*. Hydrolysates of this preparation and wheat gluten were analyzed for their contents of 17 amino acids using microbiological methods. Reasonably good agreement was found between results for wheat gluten by this procedure and those previously reported in the literature. Carob gluten differed from wheat gluten in containing much more arginine, aspartic acid, and lysine; somewhat more glycine and histidine; somewhat less cystine, glutamic acid, and phenylalanine; and much less proline. It is apparent that similar physical properties may be shared by proteins of widely varying amino acid composition.

The unique physical properties of wheat gluten are in large part responsible for the behavior of wheat flour in baked goods. Flours of other cereal grains do not possess these properties to the same degree and cannot be satisfactorily substituted for wheat flour. Variations exist in the properties of glutens depending on the type of wheat and the grade of the flour.

<sup>1</sup> Contribution from School of Nutrition, Cornell University, Ithaca, N. Y.

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<sup>2</sup> Present address: New York State Agricultural Experiment Station, Geneva, N. Y.

<sup>3</sup> Associate Professor, School of Nutrition, Cornell University.

Many workers have studied the chemistry of the wheat proteins in an effort better to explain their physical properties and account for variations in these properties. The literature in this field has been reviewed by Bailey (2). Differences have been observed in chemical composition of proteins from various flours, but correlation of these with physical behavior proved difficult.

Bienenstock *et al.* (3) have reported that proteinaceous materials possessing the physical properties of gluten may be prepared from the germ tissues of seeds of certain members of the legume family. One of these is the carob bean, *Ceratonia siliqua*, indigenous to the Mediterranean area. The carob bean pod is used in livestock feeds; the seed endosperm when ground is the product known as locust bean gum, and the seed embryo is used in Europe both in feeds and human food. The embryo contains no starch but has a very high protein content, over 50%. When the embryo is ground to a flour, it may be made into a dough with water. This dough, if carefully washed to remove non-proteinaceous materials, will yield a "gluten" which closely resembles wheat gluten in appearance, extensibility, and elasticity.

It seemed of interest to prepare some of this carob gluten and compare its amino acid composition with that of wheat gluten to ascertain whether or not two materials of such outward similarity were also similar in composition.

### Materials and Methods

Wheat flour used as a source of gluten was a commercial strong baker's patent.

Carob germ flour was prepared from carob seeds. Seed coats were removed by carbonizing in concentrated sulfuric acid, washing in water, and drying. Germ was separated from endosperm by a grinding and sifting procedure, taking advantage of the fact that the endosperm was very hard and tough, while the germ was much more friable. The germ was finally ground and bolted through a 10 xx silk bolting cloth. The germ flour recovered comprised 22% of the weight of the seeds.

Both the wheat and carob flours were extracted for 16 hrs. with petroleum ether (b.p. 30–60°C.) in a Soxhlet extractor.

Gluten was prepared from the wheat flour by the A.A.C.C. (1) method. A similar procedure was used for the carob germ flour except that 140% absorption was required in making the dough.

Following the determination of moisture content and nitrogen (Kjeldahl) according to A.A.C.C. methods (1), hydrolysates of the carob and wheat glutes were prepared. Acid and alkaline hydrolysates were made, using 20 ml. of 10% hydrochloric acid per gm. of sample for the former and 20 ml. of sodium hydroxide per gm. of sample for

the latter. The samples varied in weight from 1–1.5 gms.; the dry weight, however, was from 0.3–0.5 gms. Hydrolysis was carried out in sealed ampules at 15 lbs. pressure for 16 hours. After neutralization, the hydrolysates were filtered or centrifuged, made to volume with distilled water (acid hydrolysate, 100 ml.; alkaline hydrolysate, 200 ml.), and stored under toluene at 2°C.

The hydrolysates were analyzed microbiologically for those amino acids indicated as being present in wheat gluten by Block and Bolling (4). The alkaline hydrolysates were employed for the assays of tyrosine and tryptophan, while the acid hydrolysates were used for the remainder of the assays. The media of Steele *et al.* (10) and Stokes *et al.* (11) were used with slight modifications. A stock vitamin mixture was used, differing slightly from that of both authors, which was as follows: thiamine·HCl—250γ, pyridoxamine·HCl—500γ, pyridoxal·HCl—500γ, Ca-dl-pantothenate—250γ, riboflavin—250γ, niacin—500γ per 250 ml. of medium.

Organisms used included *Streptococcus faecalis*, *Leuconostoc mesenteroides* P-60, and *Leuconostoc citrovorum*, 8081.<sup>4</sup> These were transferred to a liver-tryptone broth (6) 16–24 hrs. prior to inoculation of the assay. Before inoculation, the broth cultures were centrifuged at 2,500 r.p.m. for 15 mins., washed with 10 ml. of physiological saline solution, recentrifuged, and made up in a saline solution to a turbidity of 70% against distilled water. Turbidity of the inoculum was determined in the same manner as turbidity of the assays.

The assay procedure employed eight dilution levels in triplicate for the standard curve and four dilution levels, in duplicate, for each sample. After inoculation with one drop of inoculum, the assay was incubated for 16 hrs. at 37°C. The results, determined turbidimetrically at 650 mμ. using filter PC-5 in a Coleman Universal spectrophotometer, were averaged for the four dilutions and again for the duplicates. The value thus obtained is reported.

## Results

The moisture and nitrogen contents of the two glutens were as follows:

|              | Moisture | Nitrogen<br>(dry basis) |
|--------------|----------|-------------------------|
| Wheat gluten | 67.9%    | 16.1%                   |
| Carob gluten | 61.0%    | 16.3%                   |

Results of the amino acid assays, in Table I, indicate a difference in the amino acid compositions of the two glutens. This is especially true for the basic amino acids, arginine, lysine, and histidine, which are found in larger proportions in the carob gluten and the heterocyclic

<sup>4</sup> Cultures were obtained from the Dept. of Biochemistry and Nutrition, Cornell University.

TABLE I  
AMINO ACID CONTENTS OF WHEAT AND CAROB GLUTENS  
BY MICROBIOLOGICAL METHODS

| Amino acid    | Wheat gluten <sup>1</sup> | Carob gluten <sup>1</sup> | Organism | Medium |
|---------------|---------------------------|---------------------------|----------|--------|
| Glycine       | 2.8                       | 4.1                       | C        | II     |
| Leucine       | 5.6                       | 5.0                       | B        | I      |
| Threonine     | 2.1                       | 2.6                       | B        | I      |
| Alanine       | 1.7                       | 2.1                       | A        | II     |
| Isoleucine    | 3.6                       | 3.2                       | B        | I      |
| Valine        | 3.5                       | 3.5                       | B        | I      |
| Phenylalanine | 4.4                       | 2.7                       | C        | I      |
| Tyrosine      | 3.4                       | 2.2                       | B        | I      |
| Cystine       | 2.2                       | 1.2                       | C        | II     |
| Methionine    | 1.3                       | 0.6                       | B        | I      |
| Glutamic acid | 28.6                      | 20.2                      | C        | II     |
| Aspartic acid | 2.7                       | 6.1                       | C        | II     |
| Arginine      | 3.3                       | 12.8                      | B        | I      |
| Lysine        | 1.4                       | 4.3                       | C        | II     |
| Histidine     | 1.9                       | 2.5                       | B        | I      |
| Proline       | 10.7                      | 2.6                       | C        | II     |
| Tryptophan    | 0.9                       | 0.6                       | B        | I      |

A—*Leuconostoc citrovorum*, 8081

B—*Streptococcus faecalis*

C—*Leuconostoc mesenteroides*, P-60

I—Stokes, *et al* (11)

II—Steele, *et al* (10)

<sup>1</sup> g./16.0 g. nitrogen.

TABLE II  
COMPARISON OF WHEAT GLUTEN COMPOSITION WITH  
VALUES REPORTED IN THE LITERATURE

| Amino acid <sup>1</sup> | Present investigation | Block and Bolling (4) | Padoa (7) |                   |
|-------------------------|-----------------------|-----------------------|-----------|-------------------|
| Alanine                 | 1.7                   | 5                     | 5.0       | 3.5 <sup>2</sup>  |
| Arginine                | 3.3                   | 3.9                   | 1.9       |                   |
| Aspartic acid           | 2.7                   | 10                    | 9.6       |                   |
| Cystine                 | 2.2                   | 1.7                   | 1.4       |                   |
| Glutamic acid           | 28.6                  | 27                    | 26.8      |                   |
| Glycine                 | 2.8                   | 9                     | 8.6       | 1.56 <sup>3</sup> |
| Histidine               | 1.9                   | 2.2                   | 0.97      |                   |
| Isoleucine              | 3.6                   | —                     | —         |                   |
| Leucine                 | 5.6                   | —                     | 8.60      |                   |
| Lysine                  | 1.4                   | 1.9                   | 1.2       |                   |
| Methionine              | 1.3                   | 3                     | —         | 0.93 <sup>4</sup> |
| Phenylalanine           | 4.4                   | 5.5                   | 4.08      |                   |
| Proline                 | 10.7                  | 10                    | 8.05      |                   |
| Threonine               | 2.1                   | 2.5                   | —         |                   |
| Tryptophan              | 0.9                   | 1.0                   | 1.24      |                   |
| Tyrosine                | 3.4                   | 3.8                   | 1.34      |                   |
| Valine                  | 3.5                   | —                     | 3.27      |                   |

<sup>1</sup> g./16.0 g. nitrogen.

<sup>2</sup> Sauberlich and Baumann (9).

<sup>3</sup> Reisen, Schweigert, and Elvehjem (8).

<sup>4</sup> Greenhut, Schweigert, and Elvehjem (5).



amino acids, proline and tryptophan, present to a greater extent in wheat gluten.

A comparison of amino acid values found for wheat gluten with those previously reported in the literature (4), (5), (7), (8), (9) is given in Table II. In making this comparison, several factors should be borne in mind. Many of the values (4), (7) were determined by chemical analyses rather than by microbiological assay. Others (5), (8), (9) were obtained by microbiological assays with titration of acidity rather than turbidity as the measure of growth. Discrepancies among assays may also result from variations in raw material, poor recovery, and destruction or racemization of amino acids during hydrolysis. Poor agreement (low values) may be noted for aspartic acid, alanine, and glycine. Otherwise the values found by the procedures employed check well with those reported by other workers.

### Discussion

The preparation of a material having the peculiar physical properties characteristic of gluten from a source other than wheat flour is of considerable interest in that it offers unique possibilities for fundamental studies on the factors responsible for these physical properties and the manner in which they may be modified by various treatments. That the formation of a glutinous structure in wheat flour dough is dependent on proper physical manipulation is generally recognized. Similar observations were made with the carob germ flour. Obtaining a strong, coherent gluten from this product required that the flour be sufficiently finely ground, that the proper amounts of water, time, and physical working be employed in preparation of the dough.

Gluten is by nature a heterogeneous mixture. Attempts to purify it by even rather mild treatments may greatly modify its properties. By comparing the compositions of glutens from different sources, some insight may be gained into which are the essential and which the non-essential constituents.

Microbiological methods for determination of amino acids cannot be regarded as perfected. Nevertheless these methods are sufficiently accurate to have become exceedingly valuable tools particularly for comparing the approximate compositions of proteins.

Since the amino acid composition of carob gluten differs quantitatively from that of wheat gluten it appears that a highly specific amino acid composition is not essential for a protein to exhibit glutinous properties. This does not, however, rule out the possibility that such properties reflect the presence of certain types of amino acid groupings with others acting as diluents having no specific influence on the physical properties.

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AN ELECTROPHORETIC ANALYSIS OF SOYBEAN PROTEIN<sup>1, 2</sup>D. R. BRIGGS and ROBERT L. MANN<sup>3</sup>

## ABSTRACT

Electrophoresis patterns for water extracts of defatted soybean meal, containing 95% of the total nitrogen, disclosed the presence of at least seven electrophoretically distinct proteins. "Glycinin," the globulin commonly considered to be the principal protein of soybeans, was found to be a mixture of components which constituted about 75% of the total soybean protein.

The composition of the globulin preparations, as shown by electrophoresis, varied considerably depending on the method of isolation. An electrophoretically homogeneous protein representing 60% of the globulin fraction was precipitated by cooling a water extract of the meal, the precipitation being enhanced by the addition of a small amount of calcium chloride. Its isoelectric point, as determined by microelectrophoresis, was pH 5.4. Solubility experiments with this protein fraction indicated it to be non-homogeneous by this criterion.

Osborne and Campbell (9) gave the name *glycinin* to that protein fraction which precipitated when a 10% sodium chloride extract of

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<sup>2</sup> Contents of this paper constitute a part of a thesis submitted by Robert L. Mann to the Graduate Faculty of the University of Minnesota in partial fulfillment of the requirements for the Doctor of Philosophy degree, June 1949.

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defatted soybean meal was dialyzed against water. They also reported the separation of three other proteins. Jones and Csonka (6) obtained five proteins by fractionation with ammonium sulfate. The fraction precipitable from a 10% sodium chloride extract at 55% saturation with ammonium sulfate resembled the glycinin of Osborne and Campbell. Its isoelectric point was pH 5.2. Earlier (2), glycinin prepared by the method of Osborne and Campbell was reported to have an isoelectric point of pH 4.7. Hartman and Cheng (3) prepared a "purified glycinin" and found the isoelectric point to be pH 5.02.

Smiley and Smith (1) showed that the nitrogen content of 16 samples of "glycinin" prepared by several methods ranged from 15.68% to 17.74%. McKinney, Sollars, and Setzkorn (8) have also recognized the fact that the composition of "glycinin" is dependent upon the method of preparation.

In view of the uncertainty which exists concerning the composition of whole soybean protein and of the anomalous behavior of glycinin when isolated by different methods, this investigation was made in an attempt to define more clearly this complicated protein system by subjecting it to electrophoretic analysis.

### Materials and Methods

**Materials.** Wisconsin Manchu soybeans grown in 1946 at the University of Minnesota were used throughout the experimental work. Samples suitable for protein extraction were prepared by grinding the beans in a Wiley mill through a 0.5 mm. screen and removing the oil by extraction with petroleum ether (boiling range 30°–60°C.) in a Soxhlet extractor. The defatted meal was air dried and stored in stoppered bottles in a cold room. A typical fat-extracted meal sample showed the following percentage composition: moisture, 9.5; nitrogen, 7.26; ash, 5.29.

**Extraction Method.** The procedure used for extracting the protein from the oil-free meal was essentially that employed by Smith *et al.* (11). Defatted meal and a portion of the solvent were placed in a centrifuge bottle and mechanically shaken for 30 min., then centrifuged until the supernatant was clear. The type of extracting medium and the ratio of meal to solvent were varied during the investigation. Depending upon the nature of the experiment, either these extracts constituted the protein solutions studied, or the protein was precipitated by one of several methods and redispersed in an appropriate solvent.

**Electrophoretic Analysis.** Protein solutions to be analyzed electrophoretically were equilibrated by dialysis for at least four days at 4°C. against several changes of a suitable buffer—toluene being used to

inhibit bacterial growth. Electrophoresis of the solutions was observed in a Tiselius apparatus equipped with a Longworth scanning device (7). On completion of a run a scanned photograph was taken. The field strength employed in all experiments was 5.5 volts/cm. and unless otherwise noted the time of each run was 6,440 sec. Since, for purposes of comparison, it is necessary in most cases to superimpose one electrophoretic pattern on another, tracings of projections of these plates are shown in this report of the work. In each figure is shown a scale equivalent to 1 cm. in the electrophoresis cell.

### Results

*Electrophoresis of The Water Extractable Protein.* Extraction of 3 g. of meal with 100 ml. of water resulted in the dispersion of 95.1% of the total nitrogen present. The solution obtained after centrifuging, although slightly cloudy, was sufficiently clear for electrophoretic analysis. Three extracts were prepared in this manner. Two were dialyzed against phosphate buffers, one at pH 7.6, 0.1 ionic strength, the other at pH 6.6, 0.1 ionic strength. The third extract was equilibrated against veronal buffer at pH 9.0 and 0.09 ionic strength.

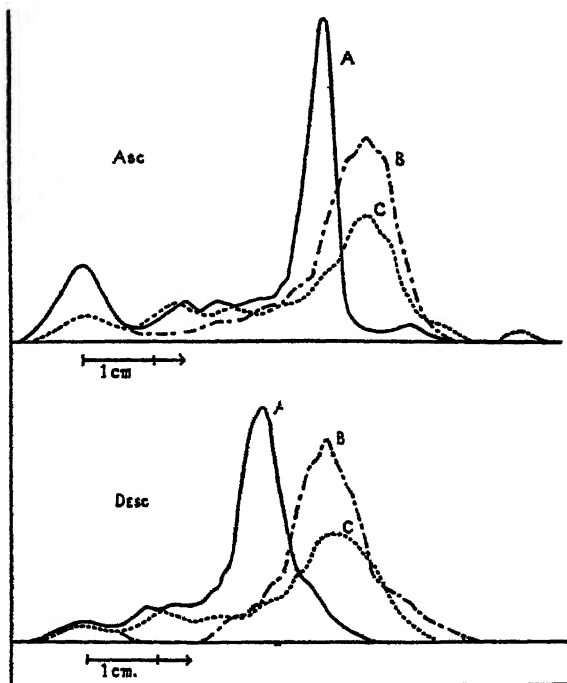


FIG. 1. Electrophoresis patterns of water extracts of deattayed soybean meal. A—pH 7.16, 6,440 seconds; B—pH 9.0, 6,440 seconds; C—pH 6.6, 10,000 seconds.

Results of electrophoresis of these preparations, each being adjusted to approximately a 1% protein concentration, are shown in Fig. 1.

These patterns reveal that while there exist at least seven electrophoretically distinct proteins in the extract, approximately 75% of the total protein is present as a fast moving fraction which appears to consist of three electrophoretically different proteins.

Since these extracts are complicated mixtures, it was desired to determine, if possible, which of the electrophoretically distinguishable

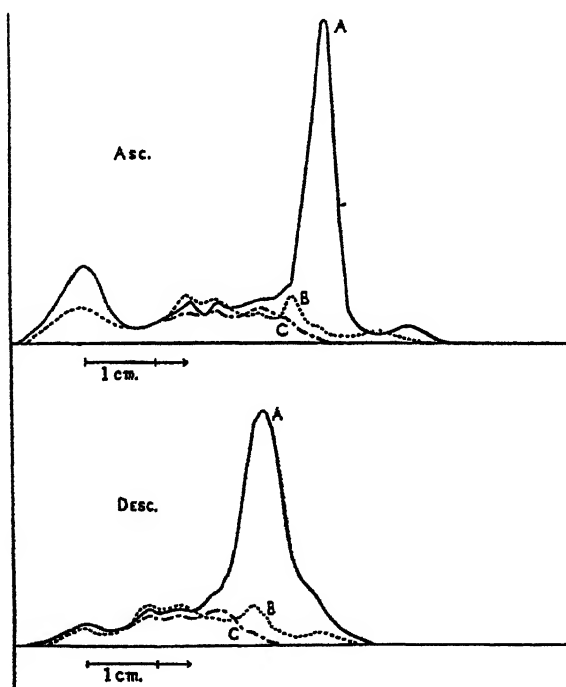


FIG. 2. Electrophoresis pattern of a whole water extract (A), compared with water extracts after elimination of "glycinin" by acid extraction (B), and by dialysis of an aqueous extract (C). The solutions were run at pH 7.6, 0.1 ionic strength.

fractions compose the protein glycinin. Glycinin was originally defined as that fraction of the total soybean protein extractable with 10% sodium chloride solution which precipitates on dialysis of a meal extract against water (9). Probably a more widely used alternate method for the preparation of the globulin is that of precipitation by adjusting an aqueous extract to pH 4.5 (4). It is apparent, then, that extraction of meal at pH 4.5 should give a dispersion containing, for the most part, those proteins other than glycinin. Accordingly, 16 g. of meal were extracted with 100 ml. of water maintained at pH 4.5

with acetic acid. This extract was dialyzed against phosphate buffer of 0.1 ionic strength and pH 7.6.

For comparison 16 g. of meal were extracted with 100 ml. of water and the extract dialyzed against running distilled water in the cold until precipitation was complete. The precipitate was removed by centrifugation and the supernatant liquid dialyzed against phosphate buffer of 0.1 ionic strength at pH 7.6.

The electrophoretic analyses of these two solutions should indicate which proteins are precipitated by acidification or by dialysis, i.e., the so-called glycinin fraction. The patterns are compared with that of a complete water extract of meal in Fig. 2.

While the patterns shown in Fig. 2 cannot be considered as yielding an accurately quantitative comparison, it is, nevertheless, evident that the globulin fraction of the soybean protein is identified with the large faster moving peak (or peaks) of the electrophoresis pattern obtained with the complete water extract of the meal.

*Electrophoretic Analysis of "Glycinin" Prepared by Several Methods.* Since the patterns of Fig. 2 indicate that glycinin may consist of more than a single component, the reported variation in composition and properties of this material may well be due to the several components being precipitated in varying proportions, depending on the method of isolation. Protein fractions were prepared by each of several methods which have been claimed to yield glycinin and these were examined electrophoretically in an effort to detect such variations in composition. The following procedures which were employed for the preparation of these samples are quite typical of those used by various investigators.

A. Water extraction, acid precipitation: Six grams of meal were extracted with 100 ml. of water. Protein was precipitated by adjusting the solution to pH 4.5 with dilute sulfuric acid.

B. Alkali extraction, acid precipitation: Six grams of meal were extracted at pH 10.5 with 100 ml. of 0.1 *N* sodium hydroxide solution. The protein was precipitated as in (A) with sulfuric acid at pH 4.5.

C. Salt extraction, precipitation by dialysis: Six grams of meal were extracted with 100 ml. of 10% sodium chloride solution and the extract was dialyzed against running distilled water until precipitation was complete. The precipitate was redispersed in 10% sodium chloride solution and again precipitated by dialysis. The dispersion and dialysis were repeated a second time. This preparation should correspond to the original glycinin of Osborne and Campbell (9).

D. Preparation according to Jones and Csonka (6): Six grams of meal were extracted with 100 ml. of water and the extract dialyzed against running distilled water. The precipitate obtained was taken

up in 10% sodium chloride solution and the glycinin precipitated by the addition of ammonium sulfate to 55% saturation.

The following amounts of protein, based on the per cent of total nitrogen extracted, were obtained as "glycinin" by each method: A, 71.6%; B, 70.0%; C, 67.3%; D, 59.0%. Each of the above preparations was dissolved in buffer so as to yield an approximately 1% protein solution and all were equilibrated against phosphate buffer of 0.1 ionic strength at pH 7.6 and examined in the electrophoresis apparatus. The Tiselius patterns are shown in Fig. 3.

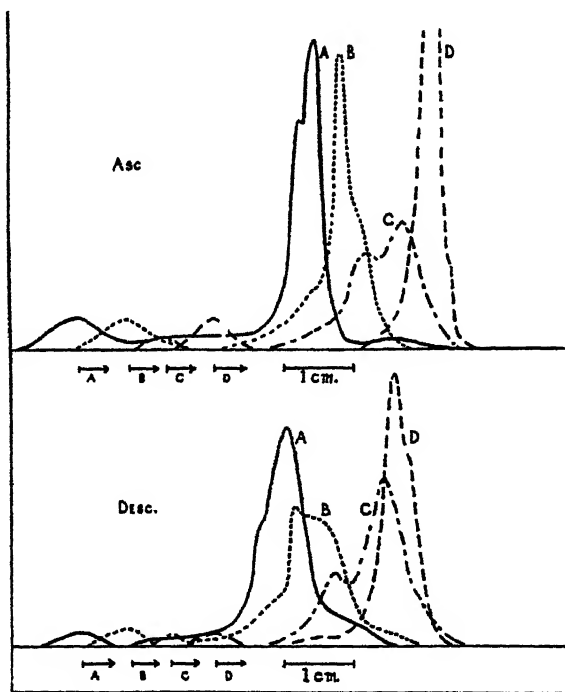


FIG. 3 Electrophoresis patterns of "glycinin" prepared by four different methods. A—method A; B—method B; C—method C; D—method D. The solutions were run at pH 7.6, 0.1 ionic strength.

The variability of "glycinin" preparations is demonstrated by these patterns. It is evident that the globulin prepared according to the method of Osborne and Campbell is electrophoretically inhomogeneous and that none of the other preparations which have been identified by the name, glycinin, is electrophoretically homogeneous or identical with the preparation of Osborne and Campbell.

The ammonium sulfate fractionation of a meal extract to give five proteins, as reported by Jones and Csonka (6), was repeated and all the fractions were found to be electrophoretically heterogeneous.

*Isolation of an Electrophoretically Homogeneous Soybean Protein.*

During some preliminary experiments it was observed that when a concentrated meal extract was cooled to 0° C. a precipitate formed which would redisperse on warming the solution again to room temperature. This precipitated material could be removed by centrifugation in the cold and, by electrophoretic comparison of aqueous extracts before and after its removal, it was shown to constitute a part of the "glycinin" fraction. In addition, the patterns gave some indication that the precipitate was composed primarily of only one of the proteins belonging to this group. This offered a clue to a possible means of separating a single protein component from a water extract.

The method used for the preparation of this cold-precipitable protein was as follows: Ten to 20 g. of meal were extracted with 100 ml. of water. The high ratio of meal to solvent was necessary since little or no precipitation occurred on cooling the aqueous extract unless the total protein concentration was somewhat greater than 1%. A centrifuge tube containing the extract was placed in ice water and allowed to stand several hours. The cloudy suspension which formed was centrifuged in the cold and the supernatant discarded. The residue was an amber colored, translucent syrup which dispersed very readily in a small quantity of distilled water. An aqueous solution of this cold-precipitated protein was clarified by centrifuging at room temperature and dialyzed against distilled water until complete precipitation of the protein resulted. The aqueous suspension of protein was either dried *in vacuo* from the frozen state or the protein was removed by centrifugation, dehydrated by washing with methyl alcohol, and dried with ether. In either case the product was a white powder which was soluble in 2% sodium chloride.

Three solutions containing this protein in a concentration of 1% were prepared with the following buffers: potassium chloride-hydrochloric acid buffer, 0.1 ionic strength, pH 2.9; phosphate buffer, 0.1 ionic strength, pH 7.3; phosphate buffer, 0.1 ionic strength, pH 7.6. The patterns obtained from the electrophoretic analysis of these solutions are shown in Fig. 4. No distinguishable electrophoretic inhomogeneity of this protein fraction is apparent.

The next step in studying this apparently homogeneous fraction was to determine to what extent it occurs in a water extract of meal. In a series of extracts having a total protein concentration varying from 2% to 4%, approximately 30% of the total protein precipitated on cooling. In this concentration range, the amount precipitated appeared to be independent of the total protein concentration.

Smith, Circle, and Brothier (11) have extensively studied the peptizability of soybean protein by neutral salts and shown that in dilute



solutions there is a sharp minimum in the peptization curve which varies in degree with the kind of salt used. They found that the curves could be fairly well reproduced by the alternate procedure of starting with a water extract and precipitating the protein by the addition of increasing quantities of salt. In the present study, aqueous solutions of the cold-precipitated protein were observed to be extremely sensitive to precipitation by the addition of neutral salts and this suggested the possibility that more of the cold-precipitable protein

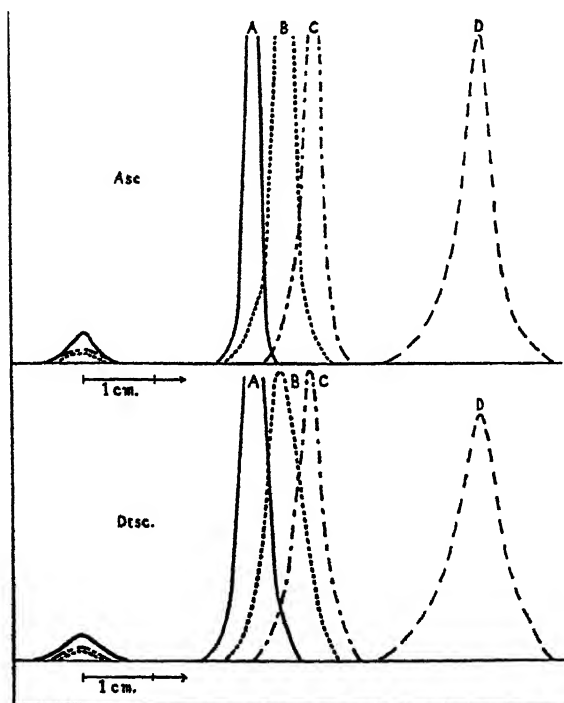


FIG. 4. Electrophoresis patterns of cold-precipitated protein run at different pH values. A—pH 2.9, 0.1 ionic strength, 6,440 seconds; B—pH 7.3, 0.1 ionic strength, 6,440 seconds; C—pH 7.6, 0.1 ionic strength, 6,440 seconds; D—pH 7.3, 0.1 ionic strength, 12,630 seconds.

might be obtained from the cooled aqueous extracts by such addition of salts. Because of the coincidence of its precipitation and peptization curves as indicated in the data of Smith, *et al.* (11), calcium chloride was chosen as the salt to be used.

Fifteen grams of meal were extracted with 100 ml. of water. The extract was cooled and the cold-precipitated protein removed by centrifugation. After warming the extract again to room temperature, calcium chloride solution was added in an amount insufficient to

bring about precipitation at room temperature. The pH was adjusted back to 6.5 (pH of the water extract) by the addition of a minute quantity of sodium hydroxide solution. The solution was then cooled again. More precipitate formed and was removed by centrifugation. This process was repeated until it became impossible to add any further calcium chloride at room temperature without precipitation. At this point it was assumed that all the cold-precipitable protein had been removed. The original cold-precipitated material plus that precipitated by adding the salt and cooling comprised 44.1% of the total soybean protein. The electrophoretic behavior of those fractions obtained on cooling after calcium chloride addition was identical in every respect with the fraction that precipitated initially from a water extract in the cold. It may be concluded that at least 44% of the total protein extractable from soybean meal by water can be isolated as an electrophoretically homogeneous material. If it is assumed that proteins precipitated by dialysis (globulins) comprise 75% of the total protein, then the cold-precipitable fraction constitutes about 60% of this fraction.

Two rather interesting possibilities were suggested by these experiments. First, Smith, *et al.* (11) found that the minimum point on the sodium chloride peptization curve showed that 48.8% of the total water extractable nitrogen was not extracted at the corresponding salt concentration. The agreement between this figure and the value 44.1% for the apparent total cold-precipitable protein obtained in the present study appears to be more than coincidental. It may be postulated that the precipitating effect of the sodium chloride is specific for the cold-precipitable protein. Some justification for this idea is found in the observation that an aqueous extract, after removal of this protein fraction, shows no precipitation on the addition of sodium chloride in any quantity. Further, the protein precipitated by sodium chloride from a water extract of meal showed electrophoretic properties identical with the cold-precipitated protein.

Second, as in the case of sodium chloride, the addition of the first increments of calcium chloride apparently causes precipitation of the cold-precipitable protein alone. However, when calcium chloride is added in an amount which will yield maximum precipitation at room temperature, the amount of protein that is precipitated represents 76% of the total extracted by water (11)—a value corresponding favorably with the percentage of total globulin components present in an extract. To determine if calcium chloride precipitates only the globulin fraction, electrophoretic patterns of an extract before and after maximum calcium chloride precipitation were made. The salt precipitation was carried out on an extract of 6.5 g. of meal with 100 ml.

of water by adding calcium chloride in small quantities until no more precipitation occurred. The coagulated protein was removed by centrifugation and a nitrogen analysis of the supernatant indicated 73.2% of the nitrogen present in the original extract had been eliminated by precipitation. The electrophoresis patterns for the proteins of the original extract and of those not precipitable by calcium chloride are compared in Fig. 5. The supposition that calcium chloride, at that concentration where it shows its maximum capacity to precipitate the

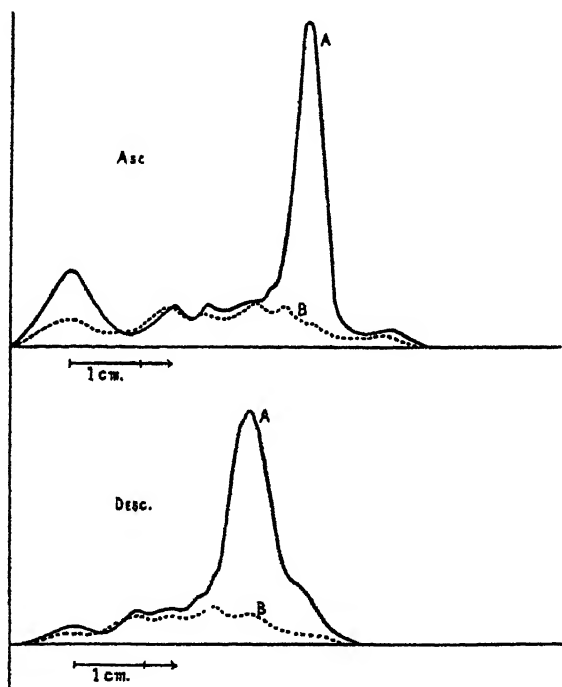


FIG. 5. Electrophoresis patterns of a water extract before (A) and after (B) maximum precipitation with calcium chloride. Run at pH 7.6, 0.1 ionic strength.

total water extracted protein, precipitates only the globulin fraction appears to be approximately correct.

The electrophoresis pattern of the whole water extract indicated the presence of three proteins in the globulin group. After removal of the cold-precipitable protein, the other two fractions may be precipitated by the further addition of calcium chloride. All attempts to fractionate these two components with calcium chloride resulted only in precipitation of mixtures of the two. Apparently both are coagulated by calcium chloride with comparable facility. The isoelectric point of

this mixture as determined by the method of microelectrophoresis was pH 4.8.

*Some Properties of the Electrophoretically Homogeneous Protein.* A detailed investigation of the optimal conditions for obtaining this protein by cooling an extract has not been carried out. It seems, however, that water is the best extracting medium. No cold precipitation will take place from a 0.85*M* sodium chloride solution, for example. The extent to which the presence of added salts affects the precipitation is not known. On the basis of purely qualitative turbidity experiments, the amount of precipitate formed is dependent on both pH and salt concentration. At pH 7.6 little cloudiness was observed at 0.2 ionic strength, but normal precipitation apparently took place at 0.1 ionic strength. At pH 6.0, however, cold precipitation occurred readily at 0.2 ionic strength.

A partial analysis of the protein showed that it contained 17.17% nitrogen, 0.73% sulfur, 0.05% phosphorus, and 0.10% ash on a dry-matter basis.

As a further criterion of purity, the solubility method (5) was applied to the electrophoretically homogeneous protein. The wet curd which resulted after dialysis of a water dispersion of the protein was suspended in a small quantity of a suitable solvent. The suspension was allowed to equilibrate with a large volume of the solvent by dialysis. Two solvents were used—1.8% sodium chloride buffered at pH 5.9 with potassium phosphate salts, 0.1 ionic strength, and phosphate buffer at pH 7.0, 0.1 ionic strength. Solubilities were determined at 5°C. and 20°C., respectively. Varying amounts of the equilibrated suspension were made to volume with the solvent and nitrogen was determined on aliquots. The solutions were rocked in a mechanical rocker until equilibrium was reached, filtered, and nitrogen was determined on aliquots of the filtrate. Fig. 6 and Fig. 7 show

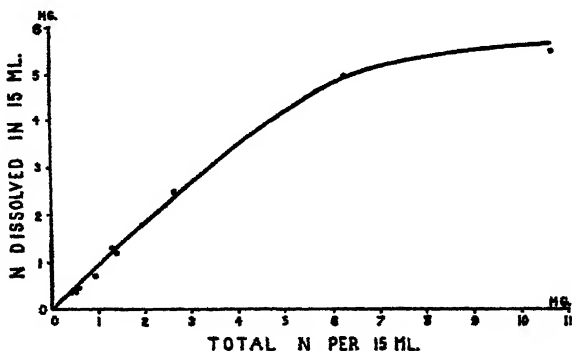


FIG. 6. Solubility of cold-precipitated protein in 1.8% sodium chloride, pH 5.9 at 5°C.

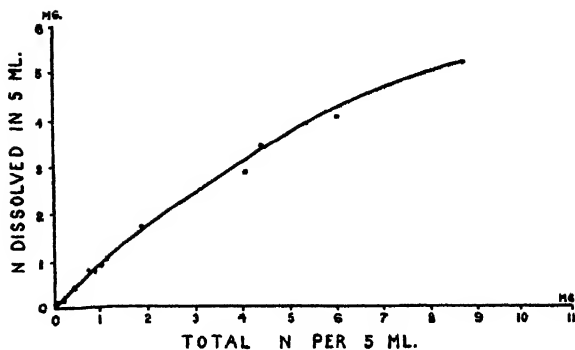


FIG. 7 Solubility of cold precipitated protein in phosphate buffer, pH 7.0, 0.1 ionic strength at 20°C

plots of total nitrogen present against nitrogen dissolved by the two solvents.

The solubility of this protein is not independent of the solid phase present and the protein cannot, therefore, be considered to be truly homogeneous.

The isoelectric point of the cold-precipitable protein was determined by the method of microelectrophoresis using a horizontal cell of the type designed by Briggs (1). Mobilities were measured by observing the movement of protein-coated quartz particles suspended in

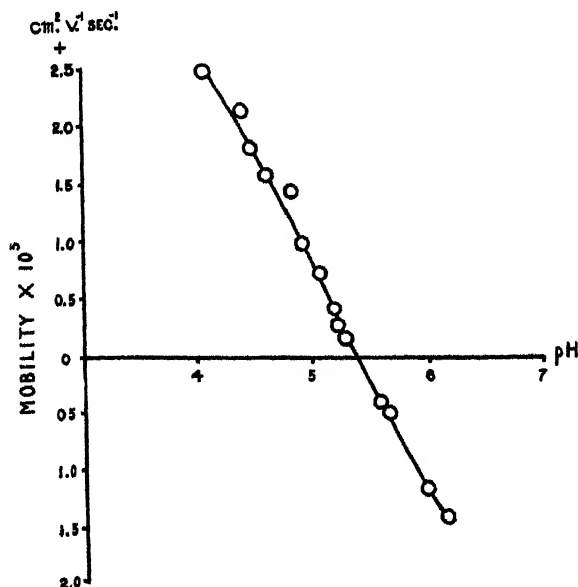


FIG. 8. pH-mobility curve for cold-precipitated protein.

acetate buffers of 0.01 ionic strength. The isoelectric point is at pH 5.4 as indicated by the pH-mobility curve shown in Fig. 8.

*The Effects of Varying the Ratio of Meal to Water During the Extraction of Protein.* Water was shown to extract 95% of the total nitrogen from meal when a ratio of 3 g. of meal to 100 ml. of water was used. In the work involving isolation of the cold-precipitable protein it was necessary to use rather high concentrations of protein which were most readily obtained by increasing the ratio of meal to water during the extraction process. However, at a meal to water ratio of 20 to 100, only about 50% of the nitrogen could be extracted. The question arose as to whether or not this 50% extract contained all the proteins in the same proportions as in the 95% extract. There was the possibility that in the 50% extract some of the proteins were preferentially retained in the meal. The answer to this question was obtained by making two extractions, one at a meal to water ratio of 3 to 100, which extracted 95% of the nitrogen, and one at a meal to water ratio of 20 to 100, which extracted 54.6% of the nitrogen. Both extracts after dialysis against buffer were adjusted by dilution with buffer solution to the same nitrogen content and examined electrophoretically. The patterns for these extracts were identical, showing the proteins to be present in the same ratio regardless of the amount extracted. Thus, the comparison of information obtained from a 50% extract, for example, with a 95% extract is justifiable.

### Discussion

Since water will extract over 95% of the total nitrogen present in soybeans, the electrophoresis patterns of such extracts present a rather complete picture of soybean protein. The Tiselius patterns of aqueous extracts adjusted to different pH values do not show significant differences other than a separation of the components to a greater or lesser degree. Fig. 1 reveals the presence in the water extract of at least seven different protein fractions at pH 7.6 in phosphate buffer. Planimeter measurement of the areas under the curves of Fig. 2 shows that about 75% of the total protein migrates as a fraction which is identified as globulin, a material which appears to be a mixture of at least three different components. It is likely that the variation in composition frequently observed in different preparations of "glycinin," i.e., soybean globulins, is a result of the precipitation of these globulin components in varying proportions. Fig. 3 also indicates that, during acid precipitation in particular (see pattern A), some of the protein components other than those of the globulin group are carried down and thus contribute to the variability in composition of the preparations compared. The most nearly homogeneous of the

preparations studied appears to be that obtained by the method of Jones and Csonka who reported its isoelectric point to be pH 5.2 (6), a value close to that of the electrophoretically homogeneous protein reported in this paper.

The effect of the presence of neutral salts on the solubility of the various soybean proteins is of considerable interest in connection with their isolation. Although sodium chloride and calcium chloride were the only salts used in this investigation, it is probable that their precipitating action is typical of the effects shown by other salts of monovalent and divalent cations respectively. That is, extraction of meal with the salts of monovalent cations at concentrations near to that of minimum peptizing capacity would not, in general, be expected to result in the dispersion of the cold-precipitable component of soybean globulin; and extraction with salts of divalent cations at corresponding concentrations would leave the entire globulin group undispersed.

No adequate explanation for the water peptizability of soybean proteins has been attempted. Such an explanation must certainly require an understanding of the action of salts on the dispersibility of the proteins in general. The precipitate, obtained on cooling an aqueous meal extract, is very soluble in water and its aqueous solutions are extremely sensitive to the addition of salts and to the removal of salts by dialysis. In view of these findings it is believed that a further investigation of the solubility properties of this fraction could contribute considerable information which would be useful in any attempt to devise an explanation of the water and salt solution peptizability of the globulins present in soybeans. The use of this system for such a study would be advantageous in that it is undoubtedly less complex than any previously described preparation of "glycinin."

It must be concluded that, on the basis of its present definition, the use of the term "glycinin" to designate any single component of the soybean protein is incompatible with the electrophoretic evidence presented in this study. The preparation of Osborne & Campbell, to which this name was originally applied, is obviously a mixture of electrophoretically distinguishable components, as is the case also with all other "glycinin" preparations studied; and the various preparations are not identical with respect to their composition ratios of these electrophoretically distinguishable components. It would appear wise to designate only the preparation of Osborne & Campbell by this term, at least until further study may indicate clearly how the term may best be redefined to designate some predominant and reasonably homogeneous globulin fraction of the soybean protein. Perhaps the cold precipitable globulin, as partially characterized in this study, may

logically, and after further characterization, inherit the name. Considerable further study of the other protein fractions of the soybean must be made, however, before any redefinition of the term should be settled upon.

### Acknowledgments

The authors are indebted to the Central Soya Company, Inc., Decatur, Indiana, who provided the fellowship during the tenure of which part of this research was conducted.

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# EFFECTS OF SOLVENT AND HEAT TREATMENTS ON SOYBEAN PROTEINS AS EVIDENCED BY ELECTROPHORETIC ANALYSIS<sup>1, 2</sup>

ROBERT L. MANN<sup>3</sup> and D. R. BRIGGS

## ABSTRACT

The effects of heat and of methanol and ethanol extraction of soybean meal on the peptizability of the soy proteins were studied electrophoretically. Hot and cold methanol or ethanol extraction of the meal reduced the subsequent extractability with water or salt solutions of all the protein components but the effect was most pronounced on the globulin components. The hot extractions were the more effective. Heating aqueous extracts of soybean meal resulted in the precipitation of protein in varying amounts, the quantity being precipitated increased with increase in temperature and with length of the heating period. This precipitation was probably the result of a heat accelerated interaction of the protein components and involved primarily those protein fractions other than the globulins. While the isolated globulins alone were unaffected in their dispersibility by heat treatment, they became increasingly more nondispersible when heated in the presence of the other protein components of the soybean extract.

The commercial value of fat-free soybean meal is dependent in part upon the extractability of the proteins which it contains. Since some methods of processing the bean subject it to contact with organic solvents and heat, it is apparent that a knowledge of the effects of these conditions on the extractability of the protein is extremely important.

For the most part, previous investigations have involved studies of the effects of heat and of methanol or ethanol extraction (1, 2, 4, 11). Although the data thus far accumulated are somewhat variable, they indicate that heat is the more important factor in reducing the amount of protein that can be subsequently extracted from soybean meal, and the effect becomes more pronounced as the moisture content of the meal is increased.

In these investigations, the so-called "denaturing" action of these agents was determined primarily by following changes in the peptizability of the protein in water, salt solutions, or alkali solutions. Such a procedure, of course, gives no information as to which components of the whole protein have become less peptizable after treatment

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<sup>2</sup> Contents of this paper constitute a part of a thesis submitted by Robert L. Mann to the Graduate Faculty of the University of Minnesota in partial fulfillment of the requirements for the Doctor of Philosophy degree, June 1949.

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nor does it indicate the nature of the changes, if any, which may be taking place in the protein molecules as a result of such treatment. It seemed that considerable information might be gained by using the method of electrophoresis in studying the effects of heat, methyl alcohol, and ethyl alcohol on soybean protein.

### Materials and Methods

*Materials.* Wisconsin Manchu soybeans grown in 1946 at the University of Minnesota were used. The beans were ground in a Wiley mill to pass a 0.5 mm screen and the oil was removed by Soxhlet extraction with petroleum ether (boiling range 30°–60°C.). The defatted meal was air dried and stored in stoppered bottles at approximately 5°C.

*Extraction Method.* The procedure used for extracting the protein with water or salt solutions was similar to that described by Smith *et al.* (9). The meal and a portion of the solvent were placed in a centrifuge bottle and mechanically shaken for 30 min., then centrifuged until the supernatant was clear. The amount of nitrogen extracted was determined by micro-Kjeldahl analysis of aliquots of the supernatant solution.

*Alcohol treatment used prior to aqueous extraction.* Defatted meal was extracted with hot or cold absolute methanol or 95% ethanol. For the hot extraction a continuous type Soxhlet extractor was used so that the temperature of the solvent in contact with the meal was 60°C. in the case of methanol and 73°C. with ethanol. The cold extractions were carried out at 20°C. in a Soxhlet extractor modified so that the solvent passed through a water-cooled coil before coming in contact with the meal. In most cases 1 g. of meal was extracted with the solvent for a definite length of time, removed from the extractor, and allowed to dry in air. The entire residue was then used for the aqueous extraction of protein.

*Heat Treatment.* A solution to be heated was placed in a test tube equipped with a condenser and immersed in an oil bath automatically maintained at the desired temperature. The time and the temperature of heat have been varied during the investigation.

*Electrophoretic Analysis.* Protein solutions to be analyzed electrophoretically were equilibrated by dialysis for at least four days at 4°C. against several changes of phosphate buffer of pH 7.6 and 0.1 ionic strength. Toluene was used to inhibit bacterial growth. Electrophoresis of the solutions was observed in a Tiselius apparatus equipped with a Longworth scanning device (7). On completion of a run a scanned photograph was taken. The field strength employed was 5.5 volts/cm. and the time of each run was 6,440 seconds. The Tiselius

patterns are shown in the figures as traced outlines in order to allow for more convenient comparisons. In each figure is shown a scale representing 1 cm. in the electrophoresis cell.

### Results

*The Effect of Methanol and Ethanol.* One gram portions of air-dried meal were extracted with hot or cold methyl or ethyl alcohol. After again drying in air each of the samples was extracted with 40 ml. of water and the amount of nitrogen in the extract was determined. The results of the experiment are shown in Table I. Hot solvent is

TABLE I  
EFFECT OF SOLVENT EXTRACTION OF SOYBEAN MEAL ON  
THE DISPERSIBILITY OF THE PROTEIN IN WATER

| <i>Extraction Solvent</i> | <i>Length of Solvent<br/>Extraction<br/>hrs.</i> | <i>Nitrogen<br/>Extracted by Water<br/>%</i> |
|---------------------------|--------------------------------------------------|----------------------------------------------|
| None                      |                                                  | 89.0                                         |
| Cold methanol (20°C.)     | 9.5                                              | 64.9                                         |
| Cold methanol (20°C.)     | 50.0                                             | 57.7                                         |
| Cold ethanol (20°C.)      | 9.5                                              | 60.0                                         |
| Hot methanol (60°C.)      | 9.5                                              | 18.2                                         |
| Hot ethanol (73°C.)       | 9.5                                              | 18.0                                         |

seen to decrease the subsequent water extractability of the proteins very markedly. Cold solvent is less effective in this regard but its effectiveness increases with the time of contact with the meal. Attempts were also made to extract the nitrogen from hot methanol or ethanol-extracted meal with sodium chloride solutions ranging in concentration from 0.02 *M* to 5 *M*. In no instance could more than 18% of the total nitrogen be extracted.

Electrophoresis patterns obtained with water extracts of hot and cold methanol extracted meals are compared in Fig. 1 with the pattern from a water extract of untreated meal. It is evident from these patterns that the globulin fraction of the total soy protein (3) is the fraction whose water extractability is decreased most markedly by the preliminary treatment of the meal with alcohol. Hot alcohol is most effective in this regard.

*The Effect of Heat.* Since the object of this experiment was to determine the direct effect of heat on the soybean proteins and not on their extractability, it was necessary to confine the heating experiment to aqueous extracts of untreated (fat extracted) meal. Therefore, the results may not be entirely comparable with the information obtained by other workers who heated the meal and then determined the peptizability of the protein with various solvents. However, it is presumed that the proteins affected and the nature of the effect should, in general, be the same in both cases.

Preliminary experiments showed that precipitation of protein occurred when an aqueous extract of meal was heated. On the basis of this observation the following possible factors in the phenomenon were investigated: (a) the dependence of the precipitation on temperature, (b) its dependence on the length of time of heating, (c) the effect that the precipitation by heating might have on the amount of electrophoretically homogeneous protein which precipitates upon cooling (3), (d) which proteins are precipitated by heating, and (e) the changes

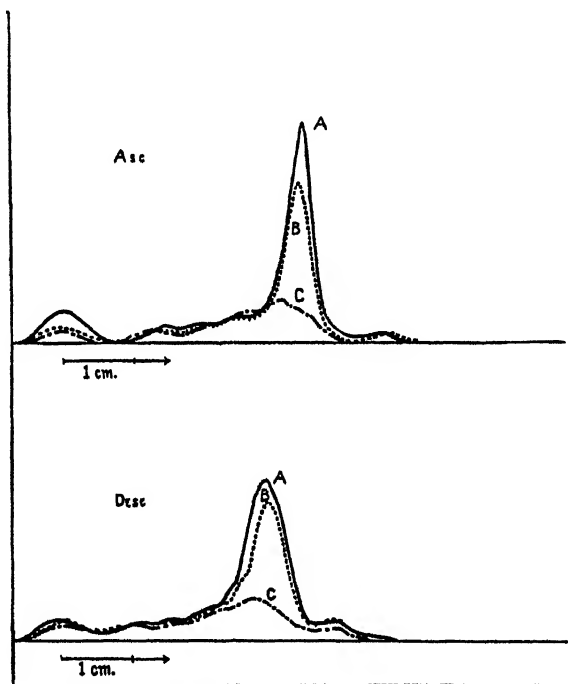


FIG. 1. Electrophoresis patterns, A—of the total protein extracted by 100 ml. of water from 3 g. of meal, B—of the total protein extracted by 100 ml. of water from 4 g. of meal previously extracted by methanol at 20°C., C—of the total protein extracted by 100 ml. of water from 15 g. of meal previously extracted by methanol at 60°C.

taking place in the electrophoretic properties of the protein molecules as a result of heat treatments.

Information concerning the first three problems was obtained from the following experiment. Twenty grams of meal were extracted with 100 ml. of water and a 10 ml. aliquot of the centrifuged extract was placed in each of a series of test tubes. Some of these tubes were heated for a two-hour period at 45°, 60°, 75°, and 90°C. respectively. The rest were heated at 75°C. for varying lengths of time from 2 hr. to 30 hr. At the completion of each heating period the precipitated

protein was removed by centrifugation and the per cent of total nitrogen which was removed from solution was determined. In none of the tubes did the pH of the solution drop more than 0.1–0.2 units below that of the original extract which was at pH 6.5. The tubes were then placed in ice water in a cold room until the cold precipitation of protein (a globulin fraction (3) is precipitated under these conditions) was complete. This precipitate was also removed by centrifugation and the percent of total (original) nitrogen lost by cold precipitation was determined. The results of the experiment are shown in Table II.

These data show that the protein component which precipitates on cooling is not precipitated by heating at 75°C. or less provided the length of the heating period does not exceed 5 hr.

To determine which protein fractions are precipitated by heat, 5 g. of meal were extracted with 100 ml. of water and the extract was heated

TABLE II  
EFFECT OF HEATING AQUEOUS MEAL EXTRACTS

| Variable Temperature   |                                         |                                                 | Variable Time   |                                           |                                                 |
|------------------------|-----------------------------------------|-------------------------------------------------|-----------------|-------------------------------------------|-------------------------------------------------|
| Temperature of Heating | Nitrogen Precipitated by Heating 2 Hrs. | Nitrogen Precipitated by Cooling Heated Samples | Time of Heating | Nitrogen Precipitated by Heating at 75 C. | Nitrogen Precipitated by Cooling Heated Samples |
| C.                     | %                                       | %                                               | <i>m s.</i>     | %                                         | %                                               |
| 45°                    | 7.0                                     | 28.3                                            | 2               | 27.3                                      | 28.9                                            |
| 60°                    | 16.3                                    | 28.8                                            | 5               | 29.3                                      | 28.9                                            |
| 75°                    | 27.5                                    | 28.7                                            | 10              | 34.4                                      | 23.5                                            |
| 90°                    | 52.2                                    | 3.5                                             | 15              | 36.6                                      | 22.2                                            |
|                        |                                         |                                                 | 20              | 38.2                                      | 19.9                                            |
|                        |                                         |                                                 |                 | 40.3                                      | 19.6                                            |

at 75°C. for 5 hr. The resulting precipitate was removed and the supernatant examined electrophoretically. The protein which precipitated was also dispersed in the standard phosphate buffer and examined electrophoretically. These patterns are shown in Fig. 2.

In a previous paper (3) it was shown that electrophoretic patterns of water extracts of meal indicated the presence of at least seven different components, three of which migrated in a group which constituted the globulin fraction. One effect of the heating becomes strikingly apparent, the precipitation by heat involves, primarily, those proteins other than globulin. The mobilities of the globulin components do not appear to be altered by the treatment. It is also seen that while, in an ordinary water extract, those proteins which are precipitated by heating show up as at least three fairly distinct peaks in the electrophoresis patterns, the pattern obtained for the heat precipitated material shows a single, fairly symmetrical peak.

Since here the globulin fraction of soybean protein is apparently not involved, it was believed that a better picture of the effect of heat could be obtained if the experiments were conducted on extracts of meal made at pH 4.5, which have been shown (3) to contain all the proteins in their usual relative proportions except the globulins which are present in a greatly diminished proportion. An observation made during preliminary experiments was that actual precipitation on heating did not occur if the aqueous protein extract was first buffered with

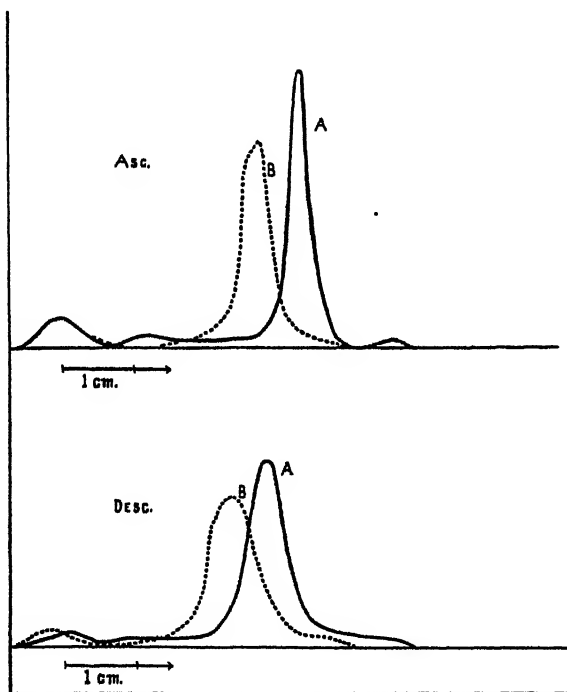


FIG. 2. Electrophoresis patterns, A—of a water extract of meal after removal of protein precipitated by heating 5 hrs. at 75°C., B—of the protein precipitated by heating a water extract of meal.

phosphate buffer at pH 7.6, 0.1 ionic strength and this proved to be useful in this connection.

Accordingly, 50 g. of meal were extracted with 200 ml. of water maintained at pH 4.5 with acetic acid. Part of this extract was dialyzed against buffer without heating. Another portion was buffered at pH 7.6 and heated in the oil bath at 75°C. for 2 hr. after which it was also dialyzed against buffer. No precipitation occurred in either portion. Both solutions were examined electrophoretically and the patterns obtained are shown in Fig. 3.

The change which occurs in the mobilities of these proteins cannot definitely be interpreted; but for the present, it may be considered as involving an interaction of these constituents under the influence of heat. The progress of this interaction could be followed by observing the electrophoresis patterns of extracts heated at 60°C. for 2 hr., for example. At this temperature and time the interaction was not complete, but part of the protein had been converted into a component migrating as a peak corresponding to the one shown for the heated sample in Fig. 3.

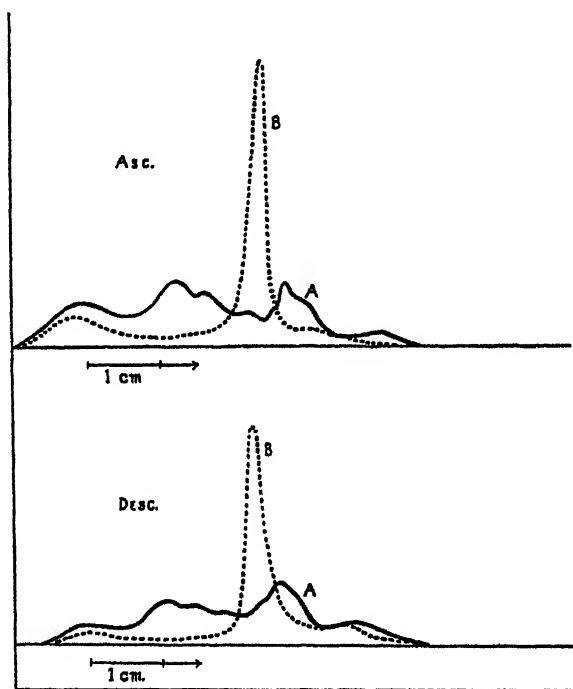


FIG. 3. Electrophoresis patterns, A—of an extract of meal made at pH 4.5, B—of the extract after heating.

As shown earlier (3), an aqueous extract of meal contains the non-globulin constituents to the extent of approximately 25% of the total protein. It is seen in Table II, however, that more protein precipitates upon heating at temperatures above 75°C., or at 75°C. when heated more than 5 hr., than can be accounted for by this 25% alone. This suggested the possibility of interaction involving, also, some of the protein of the globulin fraction. Such a reaction is implied, too, by the patterns of Fig. 3 where it is seen that the small globulin peak present in the native extract disappears on heating. In an attempt to

verify this supposition 25 g. of meal were extracted with 100 ml. of water at pH 4.5 and the extract was heated unbuffered at 75°C. for 2 hr. The resulting precipitate was removed and dispersed in buffer at pH 7.6, 0.1 ionic strength. This dispersion was diluted with an equal portion of a 1% solution of cold-precipitated protein (a globulin fraction). The mixture was examined electrophoretically before and after heating at 75°C. for 5 hr. The patterns are shown in Fig. 4.

The indication is that the minor protein components after interacting among themselves will undergo further interaction upon heating

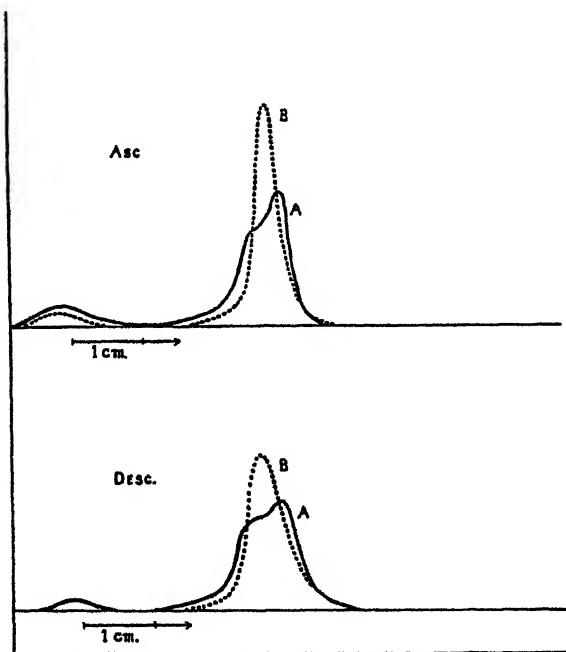


FIG. 4. Electrophoresis patterns, A—of a mixture of heat-precipitated and cold-precipitated protein, B—of the mixture after heating.

with the added cold-precipitable component of the globulin fraction. This, of course, does not exclude the possibility of reaction with the other components of the globulin fraction. The decrease in cold-precipitable protein as the amount of protein lost on heating increases (see Table II), however, indicates that the cold-precipitable component is among the first to interact with the non-globulin fractions as these undergo heat denaturation.

A rather interesting observation was made that is closely related to these heating effects. It was noted that an aqueous dispersion con-



taining all of the soybean proteins would precipitate upon standing five to seven days at room temperature. The possibility of bacterial action was eliminated, since the coagulation took place even though the extracts were protected with toluene and chloroform or after filtering through a Seitz filter and protecting against subsequent contamination. Microscopic examination, also, did not reveal the presence of any microorganisms. This coagulation was allowed to take place with several dispersions and, with only slight variation, 27% of the total nitrogen was observed to precipitate in each case. This value is in very close agreement with that obtained when extracts are heated at 75°C. for 5 hr. or less.

Experiments were made in an attempt to identify the precipitate. It was found that the cold precipitable fraction of the globulins was not affected by removal of this protein which precipitated at room temperature, and that, after its removal, no further protein could be precipitated by heating. The indication, then, is that the protein which precipitates at room temperature is the same as that which is coagulated by heating. Proof of this lies in the fact that electrophoresis patterns of an extract before and after precipitation at room temperature, as well as the pattern for the precipitate, showed that the proteins removed were the same as those precipitated by heating. The agglomeration, again, seemed to be the result of an interaction of the proteins (yielding an electrophoretically homogeneous complex). These experiments involving precipitation of aqueous extracts upon heating and at room temperature were also repeated with a 5% sodium chloride solution of the protein both before and after dialysis against sodium chloride. The results were practically identical with those obtained on water solutions. It should be pointed out that this agglomeration does not occur to a detectable degree at 4°C., and therefore does not alter the protein solutions during the four-day-dialysis period at that temperature in preparation for electrophoretic analysis.

### Discussion

*The Effect of Solvent Extraction of Meal.* Although the results show that the greatest effect of cold alcohol extraction is on the globulin fraction of soybean protein, the other components are also altered to some extent by the treatment, because the loss of water extractability cannot entirely be accounted for by the decreased area of the globulin peak. Of particular interest is the observation that extraction with hot alcohol renders the globulin fraction almost completely non-dispersible in water, whereas heating the proteins in an aqueous dispersion desolubilizes primarily those components other than globulins.

Apparently, the effect of heat is dependent upon the amount of water present.

On the basis of these experiments, the postulate (8) that alcohol extraction decreases water peptizability of the protein by removal of a natural peptizing agent would appear to be incorrect. If this were the only effect of alcohol extraction, it should still be possible to extract the globulin components with a sodium chloride solution, because these in native form are readily soluble in this solvent and do not require the presence of any other peptizing agent. The extractability of nitrogen from alcohol-treated meal with sodium chloride solutions, over and above that which is extractable with water alone, could not be demonstrated in this work. Thus, it seems that the primary effect of alcohol is to alter the globulin components in such a manner as radically to change their solubility properties.

*The Effect of Heat.* It must be emphasized that the formation of a single peak at the expense of several smaller peaks in the electrophoretic pattern of a mixture of soybean proteins is not unequivocal proof that interaction between the various component proteins has occurred. The possibility that each protein has been altered by the heat treatment so that all finally show the same electrophoretic mobility without undergoing interaction seems remote, however. Also, since the same phenomenon can apparently occur at room temperature, it would seem that it is not a result of the type of heat denaturation usually encountered in protein systems but that the heat treatment serves only to accelerate a denaturation reaction which is already able to proceed at room temperature.

Somewhat similar observations have been reported as resulting from the action of heat upon other protein mixtures in solution. Kleczkowski (6) found that on heating a mixture of the euglobulin and albumin fractions of normal rabbit serum the two proteins unite and form a complex. Van de Scheer, Wyckoff, and Clarke (10) have observed that a part of the protein of normal horse serum becomes denatured when warmed to 65°C., and a component is formed which exhibits an electrophoretic mobility approximately equal to that of  $\beta$ -globulin in the normal serum. This component arises initially at the expense of globulins but, when produced in amounts which approximate the total globulin present, incorporates considerable quantities of the albumin. The similarity between this observation and that noted upon heating a solution of the soybean proteins is noteworthy. The loss of some of the globulin fraction may be a result of its incorporation into the aggregate formed primarily by the interaction of the other proteins present in the soybean extract.

The analogy is inexact, however, in that it is the albumins of the soybean extract which undergo the primary aggregation while it is the globulin type proteins of blood serum which are the ones primarily involved when heated to 65°C. There is also the difference that the soybean albumins undergo the interaction at room temperature while the blood globulins apparently must be taken to a temperature where they become heat denatured before they show this tendency. As to the nature of the interaction, present data indicate that it involves an aggregation of primary proteins to form complexes which are still soluble or peptizable, but which contain molecules of the involved protein fractions in proportions which approximate the relative amounts of these fractions originally existent in the mixture. This is indicated by the fact that the observed mobility of the complex is always very close to the resultant mobility ( $m_R$ ) which would be calculated from the relationship,

$$m_R = \frac{C_1 m_1 + C_2 m_2 + C_3 m_3 \dots}{C_1 + C_2 + C_3 \dots}$$

where  $C_1, C_2, C_3$ , etc. represents the concentrations of the components in the uninteracted (original) mixture and  $m_1, m_2, m_3$ , etc. are their respective mobilities before interaction. In no case has there been any indication that the process involved in the formation of the complex is reversible.

The aggregation and subsequent precipitation of some of the soybean proteins, when allowed to stand in aqueous solution at room temperature, may offer an explanation for some of the changes which take place on storing soybeans. Jones and Gersdorff (5) have found that the storage of ground defatted soybean meal at room temperature resulted in a rather rapid decline in the amount of nitrogen that could be extracted by sodium chloride solutions. Although the experiments reported here involved protein solutions, it seems possible that the same type of desolubilization of protein as that which is observed to occur in solution at ordinary temperatures could occur also in stored meal because of the moisture which is present.

#### Acknowledgements

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# CEREAL CHEMISTRY

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## THE INFLUENCE OF VARIOUS TEMPERATURES, HUMIDITIES, AND OXYGEN CONCENTRATIONS ON MOLD GROWTH AND BIOCHEMICAL CHANGES IN STORED YELLOW CORN<sup>1</sup>

### GRAIN STORAGE STUDIES IX

R. A. BOTTOMLEY<sup>2</sup>, CLYDE M. CHRISTENSEN,<sup>3</sup> AND W. F. GEDDES<sup>4</sup>

#### ABSTRACT

The effects of variations in temperature and oxygen concentration in the atmosphere upon mold growth, viability, and several biochemical properties of No. 1 grade yellow dent corn stored for 12 days at different moisture contents, were determined in a factorially designed experiment. The moisture contents, which were in equilibrium with relative humidities of from 75 to 100%, varied from 17.4 to 31.2% (dry basis); the temperature ranged from 25° to 45°C. and the oxygen concentration varied from 21% to 0.1%.

Mold growth and the biochemical properties of the corn were affected most by the variations in relative humidity and least by the changes in atmospheric composition. The effect of each variable depended upon the levels of the others. As the relative humidity of the air in contact with the corn was increased from 75 to 100%, the total mold count increased logarithmically, the internal mold infection and fat acidity increased sharply, total and water-soluble nitrogen increased slightly, reducing sugars increased, while non-reducing sugars, total dry matter, and the viability of the grain decreased. The highest mold count was found at 25°C. and the highest fat acidity at 40°C.; the lowest values for these measures were obtained at 45°C. Lowering the oxygen content of the storage atmosphere from 21 to 0.1% decreased the extent of the various changes; the depression in mold count was four times greater than in fat acidity.

The nature of the microflora varied with moisture, temperature, and oxygen concentration. Corn in equilibrium with a relative humidity of 80%, and over all the atmospheric conditions employed, supported predominately *Penicillium* sp. at 25°C., *Aspergillus flavus* at 30°C., *A. glaucus* at

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35°C., and *Mucor* sp. at 45°C. The tolerance of *Penicillium* sp. and *Candida pseudotropicalis* to low oxygen tensions was marked.

Total mold count and fat acidity did not show parallel trends with variations in the conditions of storage, and a low but significant correlation,  $r = +0.20$ , was obtained. The relation is apparently influenced by the kind of mold and the length of time of development. The highest fat acidity was found at 40°C. and coincided with the highest count of *Aspergillus flavus*, although there was a higher total mold count at 25°C.

Corn deterioration could not be estimated accurately by measurement of any one of the biochemical changes which were studied, but the decrease in non-reducing sugar content was the best single index. The values varied from zero to 174 mg. of sucrose per 10 g. of corn and bore a straight-line relation to relative humidity and to the logarithms of the mold counts for the corresponding samples.

Several workers have presented evidence which indicates that microflora are primarily responsible for the respiratory activity and deterioration of grains and oil seeds when they are stored at moisture contents above that which is in equilibrium with a relative humidity of approximately 75% (11, 14, 18, 21, 24, 25, 27, 28, 30-33, 38). On the other hand, Altschul *et al.* (1, 17, 19) contend that so far as cottonseed is concerned, the enzymic activities of the seed are of primary importance.

In addition to the water activity of the substrate, its previous history, amount of impurities present, the temperature, degree of aeration, and duration of storage influence the growth of molds (3-5, 23, 34). The degree of natural microfloral infection prior to storage has been shown to be of little importance (10).

Of the various chemical changes that occur during deterioration, the increase in fat acidity has been stressed by Zeleny (40, 41). Nagel and Semeniuk (27) demonstrated that sterilized shelled corn which was inoculated with each of nine fungi and held at about 32% moisture showed considerable increases in fat acidity. Further, other biochemical changes were noted which roughly paralleled those observed in corn which had deteriorated in storage. The use of sterilized grain in studying the role of molds in seed deterioration suffers from the fact that saprobic organisms grow on it more readily than they do on viable seeds (37) as well as from the elimination of the natural competition that exists between mold species and also from the inactivation of the enzymes of the seeds themselves.

Since attempts to preserve damp wheat and corn by storing in airtight containers (6, 8, 20, 26) or by chemical treatment (10, 16, 22) have not proved successful, it appeared advisable to study further the conditions which govern the succession of, and competition between, fungi on stored corn together with the concomitant biochemical changes. This consideration prompted the present studies in which

the effect of variations in temperature, moisture content of the grain, and the atmospheric composition during storage upon the external and internal mold flora of the corn was determined in a factorially designed experiment. The corn, conditioned to moisture contents of 17.4, 18.8, 20.7, 23.6, 27.0, and 31.2% (dry basis) in equilibrium with relative humidities of 75, 80, 85, 90, 95, and 100%, respectively, was held at temperatures of 25, 30, 35, 40, and 45°C. under each of various atmospheres. The atmospheres ranged from air (21% oxygen) to one containing 20% carbon dioxide, 80% nitrogen and a trace of oxygen (0.1%), the oxygen being reduced and the carbon dioxide increased simultaneously in 5% increments. Several biochemical changes were followed, namely, changes in viability, fat acidity, total and water soluble nitrogen, reducing and non-reducing sugars, pH, and loss of dry matter of the stored corn.

### Materials and Methods

A composite sample of No. 1 grade yellow dent corn with 86% viability was selected. It was found to have 34% of the kernels internally infected with the molds *Fusarium* sp., *Penicillium* sp., and *Aspergillus glaucus* in approximately equal numbers. The total molds amounted to 12,000 per g.

A large water bath (5'-9"  $\times$  1'-8"  $\times$  1'-4" deep with automatic temperature control and a circulating pump) was fitted with manifolds so that each gas could be delivered to, and removed from, the individual samples without using a multiplicity of connecting tubes. A continuous flow of the prepared gaseous mixtures through the humidifying solutions (12) and samples was controlled by means of a reducing valve and a screw-clamp inserted between the manifold and each of the samples. The rate of flow was measured by a calibrated flow meter.

Four prepared gaseous mixtures were obtained<sup>5</sup> in cylinders containing 165 cu. ft. of gas under a pressure of 2,000 lb. per sq. in. Each gas was analyzed for its oxygen and carbon dioxide content and it was found that the nitrogen-carbon dioxide mixture contained 0.38% oxygen. By passing this mixture through an acid chromous sulphate solution as described by Stone (35) and Stone and Beeson (36), the oxygen content was kept to a maximum of 0.1% and only a slight reduction was observed in the percentage of carbon dioxide present. The average analytical values for the oxygen and carbon dioxide content of the gases used in the experiment are shown below.

The laboratory compressed air that was used was passed through a cotton filter and a calcium chloride drying tower before it was humidified.

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GAS COMPOSITION BY VOLUME

| Gas | Oxygen | Carbon Dioxide | Nitrogen<br>(by difference) |
|-----|--------|----------------|-----------------------------|
|     | %      | %              | %                           |
| I   | 14.42  | 4.83           | 80.75                       |
| II  | 9.90   | 10.12          | 79.98                       |
| III | 4.88   | 15.66          | 79.46                       |
| IV  | 0.10   | 21.33          | 78.57                       |
| Air | 20.95  | 0.01           | 79.04                       |

Each gas was brought to each of 75, 80, 85, 90, 95, and 99–100% relative humidity by bubbling through sulphuric acid solutions of appropriate density as determined by extrapolation of the data given by Wilson (39).

*Chemical Analyses.* Unless otherwise stated, all analyses were performed upon a sample of air-dried corn ground in a Wiley mill so as to pass a 0.5 mm. screen. To prevent contamination of one sample by another, the Wiley mill was carefully cleaned between each sample and the first portion ground was discarded.

The moisture content of the corn was determined using the 130°C., 1 hour, air-oven method described in Cereal Laboratory Methods (2). When the moisture content was above 17% (dry basis) the two-stage procedure was followed, the corn being air-dried before grinding. The moisture contents have been expressed on a dry-basis thus enabling the actual changes in water content to be observed readily.

Water-soluble nitrogen was determined by extracting a 3.0 g. sample with 100 ml. of toluol-saturated water for 16 hr. at room temperature. The extraction flasks were vigorously shaken at the beginning and at 30 min. before the end of this period, the suspension filtered and nitrogen determined in a 75.0 ml. aliquot by the Kjeldahl method (2). The total nitrogen was determined directly on the ground corn using a 1.0 g. sample.

Fat acidity was determined according to the method given in Cereal Laboratory Methods (2). Care was taken to measure the fat acidity within 2 hr. of grinding the samples, which in the interim, were kept at 4°C.

Reducing and non-reducing sugars were estimated according to the procedure given in Cereal Laboratory Methods (2) for flour analysis. The expression of the results as maltose and sucrose respectively does not infer that these are necessarily the sugars present in the corn.

The pH of the samples was determined by shaking 1.0 g. of the ground meal with 10.0 ml. of freshly redistilled water, allowing to stand for 30 min., decanting and determining the pH of this supernatant liquor with a glass electrode.

*Seed Viability, Internal Infection, and Mold Count.* The percentage viability and the extent of internal infection were determined on 50 seeds which had been surface-disinfected by washing in a 1.5% solution of sodium hypochlorite for 2 min., placed on agar in petri dishes and allowed to stand for 5 to 7 days. The percentage viability of the seed determined in this manner, agreed closely with that reported by the Minnesota State Seed Testing Laboratory.

The mold count was determined according to the method described by Christensen (9). It is essentially a measure of the number of viable spores. The writers recognize that under some circumstances fungi may grow vigorously without sporulating.

*Gas Analysis.* To ensure that the various gases were passed through the samples at a rate such that the respiration had a negligible effect upon gas composition, frequent gas analyses were necessary. Samples were taken from the effluent manifold and the carbon dioxide and oxygen content were determined with a Haldane-Henderson gas apparatus as described by Peters and Van Slyke (29).

*Experimental Procedure.* Six 900 g. lots of corn were each brought to the required moisture contents by adding the necessary amount of water, the additions being accompanied by thorough shaking of the sample. For those samples requiring more than 50 ml. of water, the additions were made in two lots with a 2 hr. interval between them. The corn was then held in closed containers for 24 hr. at 4°C.

The samples were then removed, allowed to come to room temperature, thoroughly mixed, and from each, subsamples were weighed out so as to give five lots of approximately 125 g. of dry matter. At this stage, a 10 g. sample was removed for a moisture determination as a check on the conditioning treatment.

The samples were contained in bottles of approximately 300 ml. capacity, closed with wired-in two-hole rubber stoppers each fitted with an outlet-tube and an inlet-tube reaching practically to the bottom of the sample. Immediately after connecting the sample bottles to their respective humidifying solutions (12) and to the outlet manifold, the gases were passed through at a rapid rate for 30 min. to displace all air. The rate of flow was then reduced to the minimum necessary to maintain a constant composition as determined by periodic analysis of the effluent gas.

For each temperature, 30 corn samples were tested, i.e., samples at each of six humidities for each of the five gases. The order in which the different temperatures were employed was determined by random selection.

Each trial lasted for 12 days. During this time the samples were inspected regularly and the appearance and extent of mold growth

noted. Whenever the corn appeared to be "packing," the sample bottle was shaken vigorously so that channeling of the incoming gas would be avoided so far as was possible.

At the conclusion of the trial, the samples were removed from the water-bath, transferred to tared new paper bags,<sup>6</sup> and weighed. Enough corn (about 35 g.) was removed for viability and internal infection tests and the weighed remainder was air-dried at room temperature for 2 to 3 days. Drying was assisted by means of a 12 in. diameter fan. After noting the air-dry weights, analyses were made as described earlier and all results were calculated on a moisture-free basis.

*Analysis of Experimental Data.* The analysis of a total of 150 samples yielded ten sets of data (mold population, germination, internal infection, fat acidity, total and water-soluble nitrogen, reducing and non-reducing sugars, pH, and loss of dry matter). These, with the exception of the results for germination and extent of internal infection, were subjected to analyses of variance (13, 15).

To study the significance of the difference between mean values for any one variable over all conditions of the other variables employed, a different error variance was used for each main effect (temperature, atmospheric composition, and relative humidity). For example, to obtain the error variance ("E<sub>mt</sub>") to test the significance of the difference due to temperature (over all humidities and atmospheres), the sums of squares for the interactions involving temperature were added to that for the second order interaction. Then, by allowing for the degrees of freedom involved, the mean square was obtained for "E<sub>mt</sub>." From this value the standard error was calculated and by applying the "t" test the difference required for significance between mean values at different temperatures was obtained at the 5% and 1% point. The value "E<sub>mt</sub>" was also used to test the significance of the main temperature effect since it was a more severe test than the use of the second order interaction. Similarly, the interactions involving atmosphere and humidity were used in conjunction with the second order interaction to calculate the error variances ("E<sub>ma</sub>" and "E<sub>mh</sub>") to be applied to the mean values for atmosphere and humidity and for the main effects, atmosphere and humidity, respectively. The second order interaction was used as error to measure the significance of the first order interactions. Several of the significant interactions have been graphed and included in the text. It should be remembered that each point in the temperature-atmosphere interaction graphs represents the mean of six values, while in the other interaction graphs each point represents the mean of five values.

<sup>6</sup> Tests proved that these bags were free from molds.

The co-variance of mold population and fat values was determined both for the total variation and its several components.

## Results

The analyses of variance are summarized in Table I<sup>7</sup> whereas the mean values for the analytical data obtained on corn stored under various temperatures, atmospheres, and relative humidities, together with the differences required for significance are given in Tables II, III, and IV, respectively.

**Mold Count.** In general, the mold count increased with increasing relative humidity and decreased as the oxygen content of the at-

TABLE I

THE EFFECTS OF VARIATIONS IN TEMPERATURE, ATMOSPHERIC COMPOSITION, AND HUMIDITY ON STORED CORN ANALYSIS OF VARIANCE

| Source of Variation               | D.F. | Mean Squares                    |                             |                      |                            |                                      |                                     |                          |
|-----------------------------------|------|---------------------------------|-----------------------------|----------------------|----------------------------|--------------------------------------|-------------------------------------|--------------------------|
|                                   |      | Molds<br>× 10 <sup>-3</sup> /g. | Fat<br>Acidity <sup>1</sup> | Total<br>Nitrogen    | Water-<br>sol.<br>Nitrogen | Reduc-<br>ing<br>Sugars <sup>2</sup> | Non-<br>red.<br>Sugars <sup>3</sup> | Loss in<br>Dry<br>Matter |
|                                   |      |                                 | %                           | %                    | %                          | %                                    | %                                   | %                        |
| Temperature (T)                   | 4    | 198,009,427 <sup>*</sup>        | 21,062 <sup>**</sup>        | 0.0408 <sup>**</sup> | 0.0111 <sup>**</sup>       | 6,281 <sup>**</sup>                  | 6,193 <sup>**</sup>                 | 14.77 <sup>**</sup>      |
| Atmosphere (A)                    | 4    | 257,108,721 <sup>**</sup>       | 27,134 <sup>**</sup>        | 0.0021 <sup>**</sup> | 0.0068 <sup>**</sup>       | 508 <sup>**</sup>                    | 1,715 <sup>**</sup>                 | 9.16 <sup>**</sup>       |
| Humidity (H)                      | 5    | 499,495,329 <sup>**</sup>       | 86,818 <sup>**</sup>        | 0.0174 <sup>*</sup>  | 0.0559 <sup>*</sup>        | 7,365 <sup>**</sup>                  | 59,042 <sup>**</sup>                | 83.06 <sup>**</sup>      |
| T × A                             | 16   | 79,743,672 <sup>**</sup>        | 1,550 <sup>**</sup>         | 0.0008 <sup>*</sup>  | 0.0011 <sup>**</sup>       | 1,057 <sup>**</sup>                  | 390 <sup>**</sup>                   | 0.86 <sup>**</sup>       |
| T × H                             | 20   | 116,348,827 <sup>**</sup>       | 6,281 <sup>**</sup>         | 0.0007 <sup>*</sup>  | 0.0023 <sup>**</sup>       | 2,200 <sup>**</sup>                  | 1,032 <sup>**</sup>                 | 7.90 <sup>**</sup>       |
| A × H                             | 20   | 180,186,163 <sup>**</sup>       | 6,558 <sup>**</sup>         | 0.0007 <sup>*</sup>  | 0.0069 <sup>**</sup>       | 438 <sup>**</sup>                    | 156 <sup>**</sup>                   | 3.84 <sup>**</sup>       |
| Error (E)                         | 80   | 70,081,237 <sup>*</sup>         | 679 <sup>*</sup>            | 0.0040 <sup>*</sup>  | 0.0005 <sup>*</sup>        | 85 <sup>*</sup>                      | 241 <sup>*</sup>                    | 0.93 <sup>*</sup>        |
| Total                             | 149  |                                 | 6,462 <sup>*</sup>          | 0.0022 <sup>*</sup>  | 0.0040 <sup>*</sup>        | 942 <sup>*</sup>                     | 252 <sup>*</sup>                    | 5.60 <sup>*</sup>        |
| (T × A) + (T × H) + E (= Emt) 116 |      | 79,391,157 <sup>*</sup>         | 1,765 <sup>*</sup>          | 0.0005 <sup>*</sup>  | 0.0009 <sup>*</sup>        | 583 <sup>*</sup>                     | 398 <sup>*</sup>                    | 2.12 <sup>*</sup>        |
| (A × T) + (A × H) + E (= Ema) 116 |      | 90,397,594 <sup>*</sup>         | 1,813 <sup>*</sup>          | 0.0005 <sup>*</sup>  | 0.0017 <sup>*</sup>        | 280 <sup>*</sup>                     | 247 <sup>*</sup>                    | 1.42 <sup>*</sup>        |
| (H × T) + (H × A) + E (= Emh) 120 |      | 96,143,323 <sup>*</sup>         | 2,593 <sup>*</sup>          | 0.0005 <sup>*</sup>  | 0.0019 <sup>*</sup>        | 496 <sup>*</sup>                     | 359 <sup>*</sup>                    | 2.58 <sup>*</sup>        |

<sup>1</sup> Milligrams of potassium hydroxide per 100 g. corn, dry basis.

<sup>2</sup> As milligrams of maltose per 10 g. corn, dry basis.

<sup>3</sup> As milligrams of sucrose per 10 g. corn, dry basis.

mosphere was lowered and as the temperature was raised from 25°C. to 30°C. or higher increments. Variations in relative humidity had the greatest effect, the mold count increasing more than 300 fold with an increase in the relative humidity from 75% to 100%. The highest mold count, 121,000,000 per g., was encountered in the sample stored under air at 100% relative humidity and 25°C. There was a significant interaction between atmosphere and humidity which was mainly due to the low mold counts obtained in the absence of oxygen (Fig. 1).

<sup>7</sup> The pH data are not shown because the variations obtained were considered of little practical importance. The maximum range in pH encountered was from 6.6 to 5.8 units, but there were no well defined trends. The maximum difference in the mean pH values obtained with the various treatments was 0.2 units.

TABLE II

(THE EFFECT OF VARIATIONS IN TEMPERATURE UPON STORED CORN,  
MEAN VALUES FOR ANALYTICAL DATA<sup>1</sup>)

| Temperature                          | Molds<br>$\times 10^{-3}/g.$ | Fat <sup>2</sup><br>Acidity | Total<br>Nitrogen | Water-sol.<br>Nitrogen | Reducing<br>Sugars <sup>3</sup> | Non-red.<br>Sugars <sup>4</sup> | Loss in<br>Dry Matter |
|--------------------------------------|------------------------------|-----------------------------|-------------------|------------------------|---------------------------------|---------------------------------|-----------------------|
| °C.                                  |                              |                             | %                 | %                      |                                 |                                 | %                     |
| 25                                   | 7,350                        | 57.6                        | 1.46              | 0.202                  | 57.4                            | 94.0                            | 0.72                  |
| 30                                   | 1,447                        | 60.5                        | 1.51              | 0.227                  | 68.3                            | 101.8                           | 0.82                  |
| 35                                   | 1,889                        | 93.5                        | 1.44              | 0.200                  | 77.7                            | 80.6                            | 1.54                  |
| 40                                   | 2,121                        | 112.1                       | 1.53              | 0.227                  | 94.6                            | 82.3                            | 2.46                  |
| 45                                   | 1,196                        | 51.1                        | 1.52              | 0.182                  | 84.8                            | 64.1                            | 1.23                  |
| Difference required for significance |                              |                             |                   |                        |                                 |                                 |                       |
| 5% Point                             | 4,552                        | 21.5                        | 0.012             | 0.015                  | 12.3                            | 10.2                            | 0.74                  |
| 1% Point                             | 6,028                        | 27.5                        | 0.016             | 0.020                  | 16.3                            | 13.5                            | 0.99                  |

<sup>1</sup> The values are the means for 30 samples stored at each temperature for 12 days; that is, for samples stored at each of six relative humidities (75 to 100%) and five atmospheres (0.1 to 21% oxygen).

<sup>2</sup> Milligrams of potassium hydroxide per 100 g. corn, dry basis.

<sup>3</sup> As milligrams of maltose per 10 g. corn, dry basis.

<sup>4</sup> As milligrams of sucrose per 10 g. corn, dry basis.

The times taken for mold growth to become visible to the naked eye are shown in Table V. Samples stored under the least favorable conditions for mold growth showed no visible molds at 12 days when the test was concluded, yet mold assays in many instances gave appreciable counts; for example, samples stored at 45°C. and 75 to 85% relative humidity, which appeared bright and normal, gave mold counts up to 218,000 per gram.

TABLE III

THE EFFECT OF VARIATIONS IN ATMOSPHERIC COMPOSITION UPON STORED CORN,  
MEAN VALUES FOR ANALYTICAL DATA<sup>1</sup>

| Atmosphere<br>Oxygen                 | Molds<br>$\times 10^{-3}/g.$ | Fat <sup>2</sup><br>Acidity | Total<br>Nitrogen | Water-sol.<br>Nitrogen | Reducing<br>Sugars <sup>3</sup> | Non-red.<br>Sugars <sup>4</sup> | Loss in<br>Dry Matter |
|--------------------------------------|------------------------------|-----------------------------|-------------------|------------------------|---------------------------------|---------------------------------|-----------------------|
| %                                    |                              |                             | %                 | %                      |                                 |                                 | %                     |
| 21                                   | 7,619                        | 81.9                        | 1.50              | 0.226                  | 78.3                            | 80.1                            | 2.04                  |
| 15                                   | 3,221                        | 98.4                        | 1.50              | 0.211                  | 81.1                            | 79.3                            | 1.43                  |
| 10                                   | 1,982                        | 88.1                        | 1.49              | 0.216                  | 73.9                            | 82.7                            | 1.56                  |
| 5                                    | 1,117                        | 84.0                        | 1.46              | 0.199                  | 70.9                            | 82.9                            | 1.16                  |
| 0                                    | 65                           | 22.4                        | 1.48              | 0.187                  | 78.9                            | 97.8                            | 0.54                  |
| Difference required for significance |                              |                             |                   |                        |                                 |                                 |                       |
| 5% point                             | 4,861                        | 21.8                        | 0.012             | 0.021                  | 8.5                             | 8.0                             | 0.61                  |
| 1% point                             | 6,432                        | 28.9                        | 0.016             | 0.028                  | 11.3                            | 10.6                            | 0.81                  |

<sup>1</sup> The values are the means for 30 samples stored under each atmosphere for 12 days; that is, for samples stored at each of five temperatures (25°-45°C.) and six relative humidities (75 to 100%).

<sup>2</sup> Milligrams of potassium hydroxide per 100 g. corn, dry basis.

<sup>3</sup> As milligrams of maltose per 10 g. corn, dry basis.

<sup>4</sup> As milligrams of sucrose per 10 g. corn, dry basis.

TABLE IV  
THE EFFECT OF VARIATIONS IN RELATIVE HUMIDITY UPON STORED CORN, MEAN  
VALUES FOR ANALYTICAL DATA<sup>1</sup>

| Relative Humidity | Molds<br>$\times 10^{-3}/g.$ | Fat<br>Acidity <sup>2</sup> | Total<br>Nitrogen | Water-sol.<br>Nitrogen | Reducing<br>Sugars <sup>3</sup> | Non-red.<br>Sugars <sup>4</sup> | Loss in<br>Dry Matter |
|-------------------|------------------------------|-----------------------------|-------------------|------------------------|---------------------------------|---------------------------------|-----------------------|
| %                 |                              |                             | %                 | %                      |                                 |                                 | %                     |
| 75                | 36                           | 23.4                        | 1.47              | 0.180                  | 58.4                            | 143.0                           | 0.28                  |
| 80                | 59                           | 24.1                        | 1.47              | 0.180                  | 68.4                            | 127.0                           | 0.30                  |
| 85                | 409                          | 35.6                        | 1.48              | 0.180                  | 74.1                            | 104.6                           | 0.32                  |
| 90                | 1,008                        | 71.4                        | 1.49              | 0.188                  | 72.9                            | 71.7                            | 0.72                  |
| 95                | 3,843                        | 143.6                       | 1.50              | 0.217                  | 77.0                            | 39.6                            | 1.58                  |
| 100               | 11,445                       | 151.6                       | 1.54              | 0.300                  | 109.0                           | 16.3                            | 4.93                  |

| Difference required for significance |       |      |       |       |      |      |      |
|--------------------------------------|-------|------|-------|-------|------|------|------|
| 5% point                             | 5,491 | 26.1 | 0.013 | 0.024 | 12.5 | 10.6 | 0.90 |
| 1% point                             | 7,266 | 34.5 | 0.017 | 0.032 | 16.5 | 14.0 | 1.19 |

<sup>1</sup> The values are the means for 25 samples stored at each relative humidity for 12 days; that is, for samples stored at each of five temperatures (25 to 45°C.) and under five atmospheres (0.1% to 21% oxygen).

<sup>2</sup> Milligrams of potassium hydroxide per 100 g. corn, dry basis.

<sup>3</sup> As milligrams of maltose per 10 g. corn, dry basis.

<sup>4</sup> As milligrams of sucrose per 10 g. corn, dry basis.

The predominant flora noted during the mold assays at the end of the 12-day storage period are reported for each corn sample in Table VI. No attempt was made to identify the various species of *Penicillium* and *Mucor* that occurred and only one mold has been recorded if it

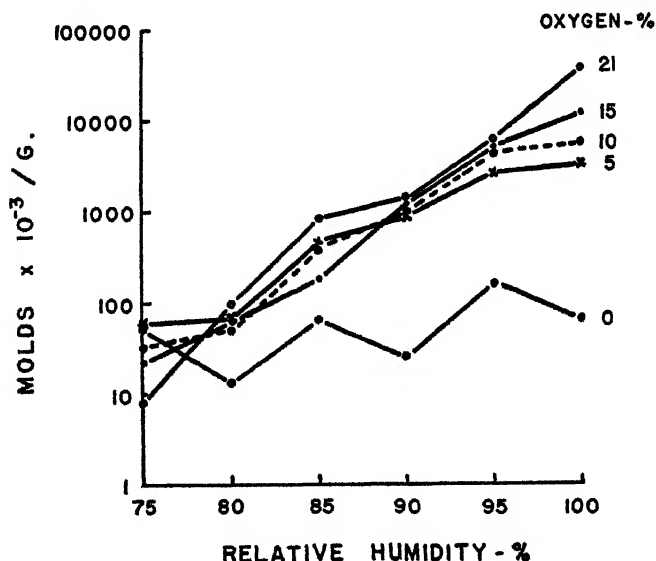


FIG. 1. The effect of atmosphere and relative humidity on the mean mold count (plotted on a logarithmic scale) of corn stored for 12 days at five temperatures (25-45°C.).

TABLE V  
TIME IN DAYS TO FIRST VISIBLE MOLD GROWTH

| Temperature | Atmos.           | Relative Humidity %            |      |      |     |    |     |
|-------------|------------------|--------------------------------|------|------|-----|----|-----|
|             |                  | 75                             | 80   | 85   | 90  | 95 | 100 |
|             |                  | First visible mold growth—days |      |      |     |    |     |
| °C.         | O <sub>2</sub> % | —                              | 5    | 4    | 4   | 3  | 3   |
| 25          | 21               | —                              | 5    | 4    | 4   | 3  | 3   |
|             | 15               | —                              | 9    | 6    | 4   | 3  | 3   |
|             | 10               | —                              | 9    | 6    | 4   | 3  | 3   |
|             | 5                | —                              | —    | 7    | 6   | 3  | 3   |
|             | 0                | —                              | —    | 10   | —   | 10 | 10  |
| 30          | 21               | —                              | 9    | 8    | 3   | 3  | 3   |
|             | 15               | —                              | —    | 8    | 3   | 3  | 3   |
|             | 10               | —                              | —    | 9    | 3   | 3  | 3   |
|             | 5                | —                              | —    | 9    | 8   | 4  | 3   |
|             | 0                | —                              | —    | —    | —   | —  | —   |
| 35          | 21               | —                              | 4    | 3    | 3   | 3  | 3   |
|             | 15               | 4                              | 4    | 3    | 3   | 3  | 3   |
|             | 10               | —                              | 9    | 4    | 3   | 3  | 3   |
|             | 5                | —                              | 9    | 4    | 3   | 3  | 3   |
|             | 0                | —                              | —    | 10   | 3   | 3  | 3   |
| 40**        | 21               | —                              | —    | 7    | 3   | 3  | 3   |
|             | 15               | —                              | 7-12 | 7-12 | 3-7 | 3  | 3   |
|             | 10               | —                              | —    | 7-12 | 3-7 | 3  | 3   |
|             | 5                | —                              | —    | 7-12 | 3-7 | 3  | 3   |
|             | 0                | —                              | —    | —    | —   | 1* | 1*  |
| 45          | 21               | —                              | —    | 9    | 6   | 2  | 1   |
|             | 15               | —                              | —    | —    | 6   | 2  | 2   |
|             | 10               | —                              | —    | —    | 2   | 2  | 2   |
|             | 5                | —                              | —    | —    | 6   | 2  | 2   |
|             | 0                | —                              | —    | —    | —   | 1* | 1*  |

\* Grains discolored and sour.

\*\* During this experiment visual examination of the samples was made only on 1, 3, 7, and 12 days.

amounted to 90% or more of the total flora. Although each corn sample usually carried a diverse mold flora, one species which changed with the storage conditions usually predominated. At 25°C., *Penicillium* sp. was in the majority although *Aspergillus glaucus* predominated in the samples stored at 85% relative humidity. In atmospheres containing 5 to 15% oxygen and a yeast-like mold, *Candida pseudotropicalis* sp. was the most prevalent in the absence of oxygen at relative humidities of 90% and higher. At 30°C., the predominant mold was *Aspergillus flavus* although in air, *Penicillium* sp. was the principal mold at 75% relative humidity, yielding in turn to *A. glaucus* and *A. flavus* as the humidity was increased. At 35°C., *A. glaucus* was the principal mold in samples stored at 80 to 90% relative humidity while *A. flavus* predominated at higher humidities. At 40°C., *A. flavus* flourished at both

TABLE VI  
PREDOMINANT MOLDS AT END OF 12-DAY STORAGE PERIOD AS PER CENT OF TOTAL MOLDS

| Temp.     | Atmos.<br>O <sub>2</sub> | Relative Humidity—%       |                   |                    |                              |                             |                    |
|-----------|--------------------------|---------------------------|-------------------|--------------------|------------------------------|-----------------------------|--------------------|
|           |                          | 75                        | 80                | 85                 | 90                           | 95                          | 100                |
| °C.<br>25 | %                        |                           |                   |                    |                              |                             |                    |
|           | 21                       | P. 60, A.gl. 30           | P. 90, A.gl. 10   | P. 45, A.gl. 45    | P. 50, A.gl. 40              | P. 70, A.fl. 20             | P. 90, A.fl. 10    |
|           | 15                       | P. 92                     | P. 90             | A.gl. 90           | P. 60, A.gl. 30              | P. 90                       | P. 80, A.fl. 20    |
|           | 10                       | A.fl. 99                  | P. 50, A.fl. 50   | A.gl. 99           | P. 55, A.gl. 40              | P. 90, A.gl. 10             | P. 90, A.fl. 10    |
|           | 5                        | A.fl. 40, P. 40,<br>M. 20 | P. 35, A.fl. 35   | A.gl. 90           | P. 50, A.gl. 45              | P. 95                       | P. 90, A.fl. 10    |
| 30        | 0                        | P. 70, A.fl. 30           | P. 55, A.fl. 45   | P. 99              | Can. 70, P. 15,<br>A.gl. 10  | Can. 99                     | P. 65, Can. 30     |
|           | 21                       | P. 90                     | A.gl. 99          | A.gl. 99           | A.fl. 60, P. 25,<br>A.gl. 10 | A.fl. 75                    | A.fl. 95           |
|           | 15                       | A.fl. 99                  | A.fl. 99          | A.fl. 99           | A.fl. 99                     | A.fl. 99                    | A.fl. 99           |
|           | 10                       | A.fl. 99                  | A.fl. 90          | A.fl. 45, A.gl. 55 | A.fl. 85, A.gl. 15           | A.fl. 80 (A.gl.,<br>P., M.) | A.fl. 99           |
|           | 5                        | A.fl. 99                  | A.fl. 99          | A.fl. 99           | A.fl. 99                     | A.fl. 99                    | A.fl. 99           |
| 35        | 0                        | A.fl. 90                  | A.fl. 90 (P., M.) | A.fl. 90 (P., M.)  | A.fl. 85 (P., M.)            | A.fl. 85 (P., M.)           | A.fl. 75, A.gl. 25 |
|           | 21                       | P. 80, A.gl. 10           | A.gl. 80, P. 20   | A.gl. 99           | A.gl. 85, A. och. 5          | A.fl. 90 (A.gl., M.)        | A.fl. 99           |
|           | 15                       | P. 50, A.gl. 50           | A.gl. 99          | A.gl. 99           | A.gl. 99                     | A.gl. 99                    | A.fl. 99           |
|           | 10                       | P. 35, A.gl. 60           | A.gl. 70, P. 30   | A.gl. 99           | A.gl. 90                     | A.gl. 65, A.fl. 30          | A.fl. 99           |
|           | 5                        | P. 65, A.gl. 30           | A.gl. 90, P. 10   | A.gl. 99           | A.gl. 80, A.fl. 15<br>(M.)   | A.gl. 30, A.fl. 60<br>(M.)  | A.fl. 99           |
| 0         |                          | P. 99                     | A.gl. 50, P. 50   | A.gl. 33, P. 67    | A.gl. 75, A.fl. 25           | A.gl. 20, A.fl. 80          | A.fl. 99           |



TABLE VI—Continued

| Temp.     | Atmos.<br>O <sub>2</sub> | Relative Humidity—%       |                           |                                 |                           |                                       |                           |
|-----------|--------------------------|---------------------------|---------------------------|---------------------------------|---------------------------|---------------------------------------|---------------------------|
|           |                          | 75                        | 80                        | 85                              | 90                        | 95                                    | 100                       |
| °C.<br>40 | %                        |                           |                           |                                 |                           |                                       |                           |
|           | 21                       | A.fl. 99                  | A.gl. 90, A.fl. 10        | A.gl. 99                        | A.gl. 90 (A.fl., M.)      | A.fl. 99 (M.)                         | A.fl. 99 (M.)             |
|           | 15                       | A.fl. 99 (M.)             | A.gl. 99                  | A.gl. 99                        | A.gl. 99 (M.)             | A.fl. 99 (M.)                         | A.fl. 99 (M.)             |
|           | 10                       | A.fl. 20, P. 75           | A.gl. 25, P. 75           | A.gl. 99                        | A.gl. 95                  | A.fl. 85                              | A.fl. 90                  |
|           | 5                        | A.fl. 90 (P., M.)         | A.gl. 95                  | A.gl. 95                        | A.gl. 75, A.fl. 15        | A.fl. 75, M. 20,                      | A.fl. 80, M. 15           |
|           | 0                        | A.fl. 75 (M.)             | A.gl. 70, A.fl. 25        | A.gl. 90 (A.fl.,<br>A.n.)       | A.gl. 85 (A.fl., M.)      | A.n. 5,<br>A.fl. 65, M. 25<br>(A.gl.) | A.fl. 80, A.n. 10<br>(M.) |
| 45        | 21                       | A.fl. 40, P. 60           | P. 40, M. 40,<br>A.fl. 20 | A.ter. 45, M. 30<br>(P., A.gl.) | M. 50, P. 50              | M. 75, P. 20                          | P. 80, M. 20              |
|           | 15                       | A.fl. 20, P. 75           | P. 85, A.fl. 15           | P. 50, A.gl. 50                 | M. 95, A.ter. 5           | M. 90                                 | P. 85, A.gl. 10           |
|           | 10                       | M. 60, P. 40              | M. 60, A.fl. 20,<br>P. 20 | M. 70, P. 30                    | M. 85 (P., Asp.)          | M. 99                                 | M. 55, P. 45              |
|           | 5                        | M. 40, A.gl. 40,<br>P. 10 | M. 45, P. 35              | M. 45, P. 45,<br>A.fl. 10       | M. 75, A.ter. 15          | M. 90, P. 10                          | P. 99                     |
|           | 0                        | M. 35, P. 55,<br>A.fl. 15 | M. 25, P. 50,<br>A.fl. 15 | M. 35, P. 35,<br>A.fl. 20       | M. 15, P. 45,<br>A.fl. 35 | M. 25, P. 55                          | M. 40, P. 45,<br>A.sp. 15 |

Abbreviations. P.—*Penicillium* sp.  
 A.gl.—*Aspergillus glaucus*  
 A.fl.—*A. flavus*  
 A.ter.—*A. terreus*  
 A.och.—*A. ochraceus*  
 A.n.—*A. niger*  
 Asp.—Unidentified *Aspergillus* sp.  
 M.—*Mucor* sp.  
 Can.—*Candida* sp.

low and high humidities, but *A. glaucus* was dominant at 80 to 90% relative humidity, inclusive. At 45°C., the incidence of *A. flavus* and *A. glaucus* was considerably reduced, *Mucor* sp. and *Penicillium* sp. taking their place.

It must be emphasized that the mold studies were made at the end of the 12-day storage period. The relative preponderance of the various species probably changed throughout the trial and mold assays made only at the end of the period may be of limited value in estimating the numbers and kinds of molds in relation to the deterioration which has occurred in the grain.

*Internal Infection of the Corn.* The effects of variations in temperature, atmosphere, and relative humidity on the percentage of corn kernels which were internally infected with molds are shown in Table VII. The nature of the predominant internal flora is recorded in

TABLE VII  
PERCENTAGE OF CORN KERNELS SHOWING INTERNAL INFECTION WITH MOLDS  
FOLLOWING STORAGE FOR 12 DAYS UNDER VARIOUS CONDITIONS<sup>1</sup>

|                            |      |      |      |      |      |      |
|----------------------------|------|------|------|------|------|------|
| Temperature, °C.           | 25   | 30   | 35   | 40   | 45   |      |
| Mean internal infection, % | 36.1 | 40.7 | 38.7 | 36.7 | 35.0 |      |
| Atmosphere—oxygen, %       | 21   | 15   | 10   | 5    | 0    |      |
| Mean internal infection, % | 46.3 | 41.9 | 37.7 | 42.8 | 17.7 |      |
| Relative humidity, %       | 75   | 80   | 85   | 90   | 95   | 100  |
| Mean internal infection, % | 1.8  | 4.9  | 14.6 | 35.2 | 81.7 | 86.3 |

<sup>1</sup> The values for internal infection recorded for each temperature, atmosphere, and relative humidity, respectively, are means for all conditions of the other two variables. For example, the data for each temperature are the means for 30 samples (five atmospheres at each of six relative humidities).

Table VIII. The results approximately parallel those obtained for total mold count.

*Corn Viability.* The viability of the corn was adversely affected by all storage conditions, but variations in oxygen concentration had less effect than increases in temperature or relative humidity (Table IX). Few samples stored at 100% relative humidity or at 45°C. were viable. The least reduction in germination occurred in the sample stored at 30°C. under 75% relative humidity and "zero" oxygen content for which a viability of 60% (a decrease of 26%) was recorded.

*Fat Acidity.* Variations in the relative humidity of the storage atmosphere had a greater effect on fat acidity formation than differences in temperature or oxygen concentration (Tables II, III, and IV). Fat acidity increased from a minimum value of 23.4 to a maximum of 151.6 as the relative humidity increased from 75 to 100% (Table II). The mean value obtained at 100% relative humidity was only a little greater than that found at 95%. In some instances a fat acidity value



TABLE IX  
THE VIABILITY OF CORN FOLLOWING STORAGE FOR 12 DAYS UNDER VARIOUS CONDITIONS<sup>1</sup>

|                      |      |      |      |      |      |     |
|----------------------|------|------|------|------|------|-----|
| Temperature, °C.     | 25   | 30   | 35   | 40   | 45   |     |
| Mean viability, %    | 20.1 | 18.9 | 9.8  | 6.0  | 0.3  |     |
| Atmosphere—oxygen, % | 21   | 15   | 10   | 5    | 0    |     |
| Mean viability, %    | 7.8  | 9.8  | 10.4 | 11.4 | 14.6 |     |
| Relative humidity, % | 75   | 80   | 85   | 90   | 95   | 100 |
| Mean viability, %    | 27.0 | 18.4 | 11.3 | 6.8  | 2.2  | 0.6 |

<sup>1</sup> The viability values recorded for each temperature, atmosphere, and relative humidity, respectively, are means for all conditions of the other two variables. For example, the data for each temperature are the mean viabilities for 30 samples (five atmospheres at each of six relative humidities). The control sample showed 86% germination and only fell to 82.5% after several months at 4°C.

was noted which was lower at 100% than at 95% relative humidity, although the samples had been held under the same conditions of temperature and atmosphere.

As the temperature increased from 25° to 40°C., there was a rise in fat acidity from 57.6 to 112.1, followed by a decrease to 51.1 at 45°C.

Decreasing the oxygen content from 21 to 5% produced no significant changes in mean fat acidity values. However, with 0.1% oxygen there was a highly significant reduction in fat acidity as compared with any of the other values.

The interactions of temperature  $\times$  atmosphere, temperature  $\times$  humidity, and atmosphere  $\times$  humidity on fat acidity are illustrated

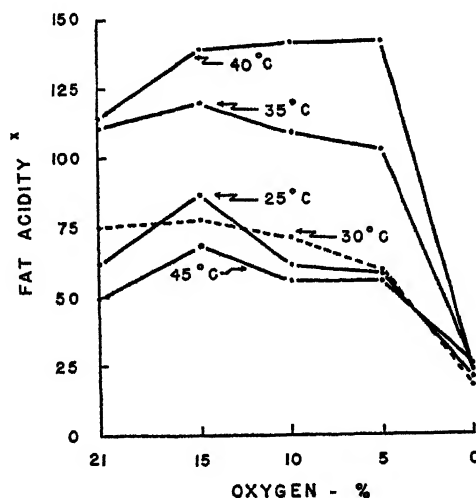


FIG. 2. The effect of temperature and atmosphere on the mean fat acidity of corn stored for 12 days at six relative humidities (75-100%). Fat acidity is expressed as mg. of potassium hydroxide per 100 g. of corn (dry basis).

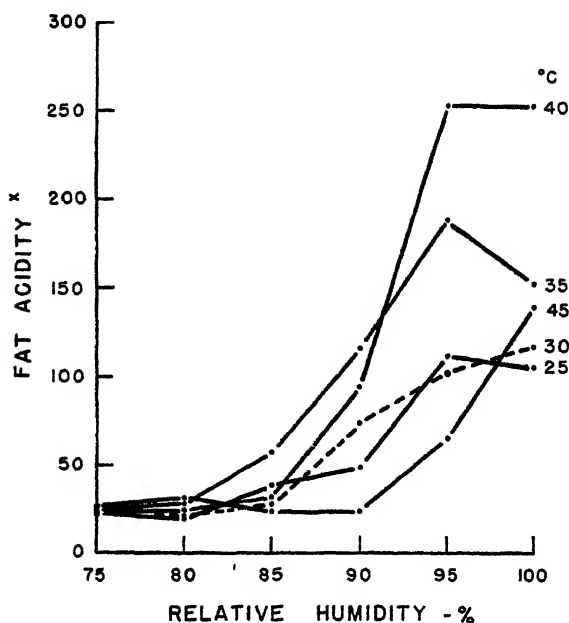


FIG. 3. The effect of temperature and relative humidity on the mean fat acidity of corn stored for 12 days under five atmospheres (0.1 to 21% oxygen). Fat acidity is expressed as mg. of potassium hydroxide per 100 g. of corn (dry basis).

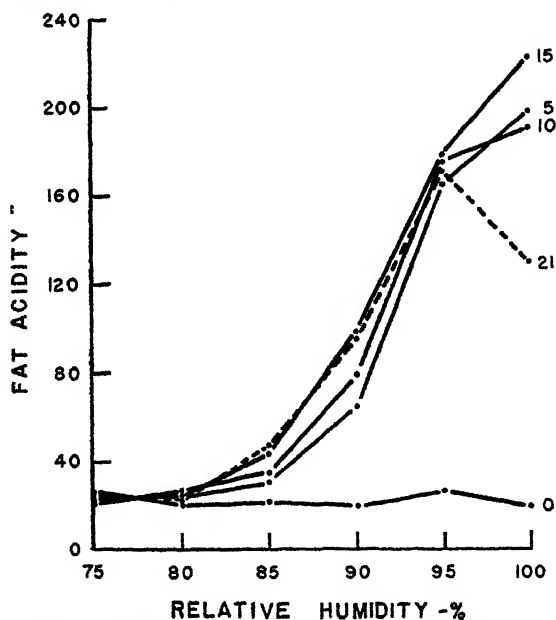


FIG. 4. The effect of atmosphere and relative humidity on the mean fat acidity of corn stored for 12 days at five temperatures (25-45°C.). Fat acidity is expressed as mg. of potassium hydroxide per 100 g. of corn (dry basis).

in Figs. 2, 3, and 4. The interaction of temperature  $\times$  atmosphere is largely due to the fact that at 40°C., the maximum acidity was recorded in 5% oxygen while for all other temperatures the maxima were reached in 15% oxygen.

The interaction of temperature  $\times$  humidity upon fat acidity is due largely to the much greater increases in fat acidity with increases in humidity from 80 to 95% at 35° and 40°C. than at other temperatures. A marked decrease in fat acidity with increase in humidity from 95 to 100% was noted at 35°C.

The anomalies in the curves from 25°C. shown in Figs. 2 and 3 may be associated with the large populations of *Penicillium* which predominated only at this temperature.

Figure 4 shows that fat acidity was uninfluenced by increasing the relative humidity unless oxygen was present. In the presence of various concentrations of oxygen, the increases in fat acidity with increasing humidity were quite uniform except that in air (21% oxygen), the maximum value was recorded at 95% relative humidity.

The correlations between the mold counts and fat acidity values obtained by an analyses of covariance are as follows:

| Component                  | <i>r</i> | Expected <i>t</i><br>at 5% point |
|----------------------------|----------|----------------------------------|
| Temperature ( <i>T</i> )   | -0.23    | 0.95                             |
| Atmosphere ( <i>A</i> )    | +0.46    | 0.95                             |
| Humidity ( <i>H</i> )      | +0.85    | 0.88                             |
| <i>T</i> $\times$ <i>A</i> | -0.03    | 0.49                             |
| <i>T</i> $\times$ <i>H</i> | -0.26    | 0.44                             |
| <i>A</i> $\times$ <i>H</i> | +0.01    | 0.44                             |
| Error                      | +0.05    | 0.22                             |
| Total                      | +0.20    | 0.16                             |

A significant, but low, positive over-all correlation existed between mold count and fat acidity. None of the several components showed a significant correlation, though that for humidity closely approached the level required for significance at the 5% point.

**Total and Water-Soluble Nitrogen Content.** The total and water-soluble nitrogen contents of stored corn were influenced by variations in the atmosphere, temperature, and relative humidity and the first order interactions were all significant. However, the changes were of a low order of magnitude. For example, an increase from 75 to 100% relative humidity was associated with an increase in the mean total nitrogen content from 1.47 to 1.54% and in water-soluble nitrogen from 0.18 to 0.30%. Changes in the atmospheric composition or temperature had even smaller effects.

**Reducing Sugars.** The reducing sugar content of the corn was markedly influenced by variations in the temperature and relative humidity of the storage atmosphere but not by changes in atmospheric

composition (Table I). The maltose values increased with increases in temperature between 25° to 40°C. and also as the humidity was raised, the maximum value being obtained for 100% relative humidity (Tables II, IV).

The first order interactions were all highly significant. That for temperature and atmosphere was derived largely from the abnormally high reducing sugar content of corn stored under 15% oxygen at 40°C. and of the corn stored under a nearly oxygen-free atmosphere at 45°C. These results are not entirely in harmony with the mold data and the question arises whether the reducing sugar values are a good index of the total metabolic activity of a changing mold flora.

The interaction of temperature  $\times$  humidity was due principally to the abnormally high reducing sugar values for the corn stored at 100% relative humidity at temperatures of 35° and 40°C. As *Aspergillus flavus* was the predominant mold present under these conditions, these high maltose values were associated with the presence of a mold known to form  $\alpha$ -amylase (7) and with conditions which favor its activity.

The interaction of atmosphere  $\times$  humidity upon the reducing sugar values of the stored corn was due largely to greater increases in the reducing sugar content of the samples stored under 15 and 21% oxygen when the relative humidity was increased from 95 to 100% than was the case for the samples stored under lower oxygen levels.

**Non-Reducing Sugars.** High values for non-reducing sugars indicate the presence of low enzyme activity and vice versa. This is the opposite of the case with the reducing sugars which have just been discussed. The maximum disappearance of non-reducing sugars at different temperatures occurred at 45°C. at which the lowest mean mold population was found. There is the possibility that the *Mucor* noted at this temperature was more active in secreting invertase than the species of molds which were predominant at lower temperatures. On the other hand, the low non-reducing sugar content may be related to bacterial activity at the higher relative humidities or to an increase in invertase activity with increase in temperature. Further investigation is necessary to provide an interpretation of these results.

Variation in atmosphere had little effect upon the quantity of non-reducing sugars found in the stored corn except when the oxygen content was lowered to 0% (Table III). At this level, the non-reducing sugars significantly exceeded the amounts at all of the other concentrations (Table III). The major difference between atmospheres in their effect upon the disappearance of non-reducing sugars occurred at 25°C. when the range in mean values was from 77.8 to 128.2 mg. per 10 g. of corn.

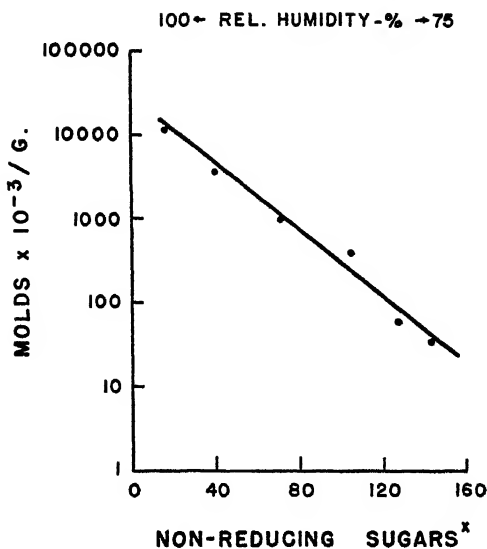


FIG. 5. The relation between mold count (plotted on a logarithmic scale) and non-reducing sugar content of corn stored under relative humidities of from 75 to 100%. Each point represents the mean of 25 values; that is, for corn samples stored at each of five temperatures (25–45°C.) and five atmospheres (0–21% oxygen). Non-reducing sugars are expressed as mg. sucrose/10 g. corn, dry basis.

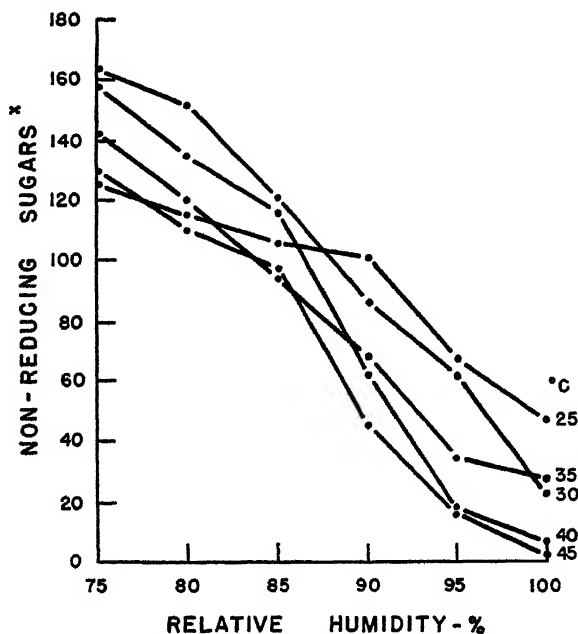


FIG. 6. The effect of temperature and relative humidity on the mean non-reducing sugar content of corn stored 12 days under five atmospheres (0.1–21% oxygen). Non-reducing sugars are expressed as mg. sucrose/10 g. corn, dry basis.



An almost linear decrease in mean non-reducing sugars from 143.0 to 16.3 mg./10 g. corn was observed with increase in relative humidity from 75 to 100% (Table IV).

When these values were plotted against the logarithms of the mold contents for the same relative humidities, the straight line shown in Fig. 5 was obtained.

The relations between non-reducing sugars and relative humidity for the various temperatures are shown in Fig. 6. The interaction of temperature  $\times$  humidity is chiefly a result of the smaller decrease in non-reducing sugar content at 25°C. with an increase in relative humidity as compared with the other temperatures.

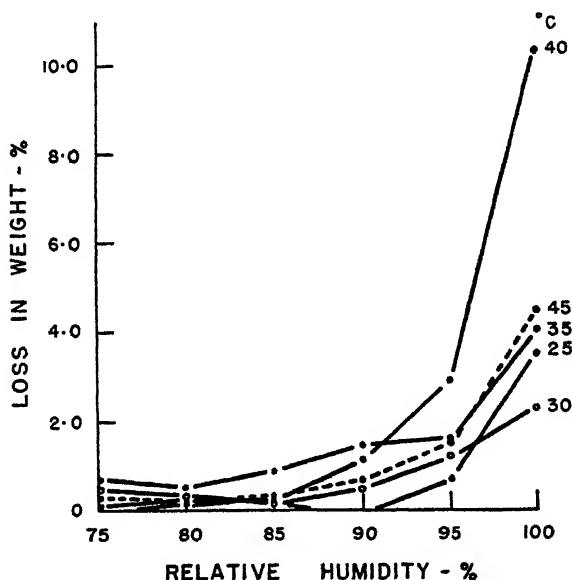


FIG. 7. The effect of temperature and relative humidity on the mean loss in weight of corn stored for 12 days under five atmospheres (0.1-21% oxygen).

*Loss of Dry Matter in Stored Corn.* Of the various conditions imposed upon the stored corn, changes in relative humidity had the greatest effect upon the loss of dry matter; the minimum loss of 0.28% occurred at 75% and the maximum 4.93% at 100% relative humidity (Table IV). As the temperature was raised from 25° to 40°C. there was a significant and progressive increase in the mean loss followed by a significant decrease when the temperature was further raised to 45°C (Table II). Variations in atmospheric composition caused smaller changes in mean loss than did variations in temperature (Table II). The maximum loss occurred when the corn was stored under air and the minimum under anaerobic conditions.

The highly significant relationships between loss of dry matter and relative humidity over the various storage temperatures and atmospheres are shown in Figs. 7 and 8, respectively.

Below 90% relative humidity, the effect of increasing the temperature from 25° to 45°C. was not marked. Above this relative humidity, and particularly at 95 and 100%, the effect of higher temperature was reflected by greater losses of dry matter. The loss sustained at 40°C. was very much larger than that occurring at the other temperatures.

Similarly, below a relative humidity of 90% the effects of variations in atmosphere or humidity upon loss of dry matter were not pronounced (Fig. 8). Above this humidity the dry matter losses became greater as the humidity was raised and the oxygen content of the storage atmosphere was increased. The maximum loss in dry

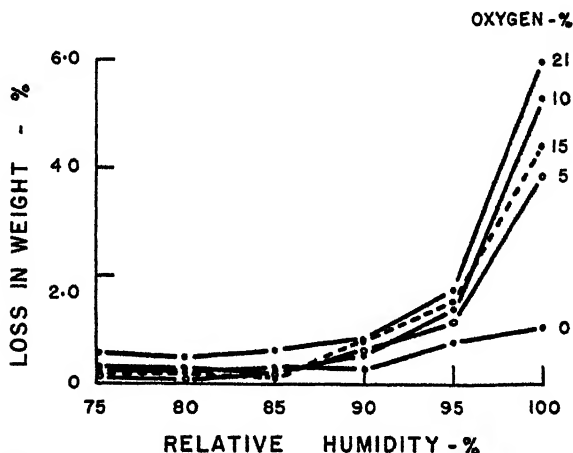


FIG 8 The effect of atmosphere and relative humidity on the mean loss in weight of corn stored 12 days at five temperatures (25 to 45°C.).

matter occurred in the sample stored under air of a relative humidity of 100% at a temperature of 40°C.

The loss in dry matter experienced in the stored corn is a result of seed respiration and/or metabolism and respiration of the molds. Actual germination of the corn was only observed in a few seeds of one sample, namely that stored under air at 30°C. and 100% relative humidity. Seed respiration could not account for the losses noted (up to 2.5%) in samples stored anaerobically, since Milner and Geddes (23) have shown seed respiration to be negligible under this condition. While increases in temperature and in the oxygen content of the atmosphere tended to increase the loss in weight and the mold population, a plot of the data indicated that there was little correlation between the two factors. This is not surprising in view of the differences

reported by Nagel and Semeniuk (27) in the ability of various molds to decompose organic matter. However, the maximum mean loss in dry matter did occur at 40°C., the temperature which supported the maximum growth of *Aspergillus flavus*, a mold known to be highly active in decomposing corn.

### Discussion

This factorial experiment was designed to give the naturally-occurring microflora on No. 1 grade yellow corn an unhindered opportunity for development under conditions of temperature and atmosphere which could occur during commercial storage. To evaluate the role of molds in the deterioration of the grain, levels of moisture were employed which were above those permitted in No. 1 grade corn. It may safely be assumed that the deterioration which followed would have been equalled, if not exceeded, in corn of naturally high moisture content.

The development of the naturally-occurring microflora of corn is dependent not only on the availability of moisture, but also on temperature and on atmospheric conditions. Thus, corn in equilibrium with a relative humidity of 80%, and over all the conditions of atmosphere employed, supported predominately *Penicillium* sp. at 25°C., *Aspergillus flavus* at 30°C., and *A. glaucus* at 35°C. Temperatures of 40 and 45°C. limited mold development, but at 45°C. *Mucor* sp. was, in general, predominant.

Essentially anaerobic conditions (about 0.1% oxygen), though greatly reducing mold development at temperatures above 25°C., failed to prevent mold growth with consequent corn deterioration at these temperatures. The tolerance of some molds, especially *Penicillium* sp. and *Candida pseudotropicalis* to these conditions was marked and indicates the impracticability of storing high-moisture grain under hermetically-sealed, anaerobic conditions.

Although a significant, positive correlation ( $r = +0.20$ ) was obtained between mold count and fat acidity, it was too low to be of practical value. Reports in the literature, including previous studies from these laboratories, have almost invariably shown that factors which favor an increase in mold population also result in a rather parallel increase in fat acidity. In fact, fat acidity has been regarded as the most valuable single index of the "commercial condition" of grain (42). The question naturally arises whether the poor correlation obtained in the present study represents the true existing relation or whether it is the result of failure of the experimental techniques to provide a reliable measure of the numbers or metabolic activities of the molds under the widely varying experimental conditions to which the corn was subjected.

The plates made to determine the extent of mold contamination actually only yield counts of the mold spores which germinated to produce individual colonies and of fragments of mycelium which produced individual colonies. Although the number of viable spores may serve as a rough criterion of the extent of mold growth as a whole, it is well-known that with some molds certain conditions which favor mycelial development do not favor sporulation. Moreover, the mold assays were made at the end of the 12-day trial and complex ecological changes in the mold flora probably occurred under some of the storage conditions. Different species of molds are known to differ greatly in the amounts of lipase and other enzymes which they produce and in their ability to metabolize such products as fatty acids and maltose. Although limitations in the methods of measuring mold activity may be in part responsible for the poor relation found between mold count and lipase activity, further research may well reveal that fat acidity is of less value as an index of grain deterioration than has been commonly supposed. Samples of grain stored over various periods of time under conditions which favor the growth of different species of molds would hardly be expected to show a close association between fat acidity and the soundness of the grain.

In the present study fat acidities were frequently lower in samples at 100% than at 95% relative humidity even though the mold counts and deterioration were greater at the higher humidity. In no instance of essentially anaerobic storage did the fat acidity exceed 40 notwithstanding mold counts of 765,000/g. and evident unsoundness of the grain. The low fat acidity may have been due to the low lipase production by the species of molds which were prevalent under the extremely low oxygen tension or to their utilization of the fatty acids which were produced. Further studies under controlled conditions of the metabolic activity of the principal molds involved in the deterioration of stored seeds are needed.

| The variations in the total nitrogen content of the stored corn are thought to be due to the removal of carbohydrates through seed or mold metabolism, the nitrogen content of the remainder increasing with increase in loss of dry matter. The trend in mean total nitrogen content for the different humidities used in the experiment parallels that for the loss in dry matter, both increasing as the humidity was raised.

| The greatest changes in the content of water-soluble nitrogen were observed at 100% relative humidity at all temperatures. While it is suspected that alterations in this factor reflect the proteolytic activity of the various molds there are some indications that factors other than mold growth may be involved.

The relatively slight changes occurring in the pH values demonstrate that this determination is of little value in the estimation of the soundness of corn. The buffer capacity of corn is sufficiently high to prevent drastic changes in acidity.

The estimation of the non-reducing sugar content was the best single index for determining the extent of deterioration which the corn suffered. In no instance was a low non-reducing sugar content found when the corn was but little damaged, as judged from all the other factors measured. The range in values was 174 to 0 mg. of sucrose per 10 g. of corn, which is sufficiently large to permit clear differentiation between various samples. The mean sucrose values showed an almost linear decrease with increase in relative humidity; they also bore a straight-line relationship with the logarithms of the corresponding mean mold populations.

#### Acknowledgments

The authors are indebted to the Quaker Oats Company, Chicago, Illinois, in providing the Fellowship under which this study was made, and in furnishing the corn used. They are also greatly obliged to Mr. J. J. Goodman, Division of Plant Pathology, University of Minnesota, for mycological work in connection with this study. The advice received from Professor H. L. Thomas concerning the statistical analyses is gratefully acknowledged.

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## THE DECOMPOSITION OF POTASSIUM IODATE DURING THE BAKING OF BREAD<sup>1</sup>

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### ABSTRACT

Potassium iodate containing radioactive iodine ( $I^{131}$ ; half-life 8.0 days) was used to determine the fate of iodate during the baking of bread. Bread samples baked from dough containing radioactive potassium iodate were extracted with ammoniacal methanol to recover iodate and iodide. Protective carriers were added to facilitate the separation of these salts, and to minimize the extent of the side reactions of these components occurring during the extraction process. Iodide and iodate fractions, fat fractions, extracted bread solids, and oven gases were examined for radioactivity. Less than 7.5% of the original 3.5-4 p.p.m. iodate is left in the baked bread. The major decomposition product is iodide.

The improving action of a small amount of potassium bromate was first reported by Kohman *et al.* (5) in 1916. The use of potassium bromate in the maturing of flour and the improving of dough has since grown into widespread practice. Potassium bromate is supplemented with potassium iodate in certain instances for developing the optimum handling and baking qualities of dough. A combination of potassium bromate and iodate is used in the dough improver marketed under the trade-mark "Fermaloid" (8).

Although the mode of action and fate of potassium bromate in the baking process has been exhaustively studied (3, 9, 10), little is known about the behavior of iodate under similar circumstances other than that it is more readily reducible than the bromate under certain conditions (11). The purpose of the experiments described below was to ascertain the fate of potassium iodate when added to bread dough as a dough conditioner during the baking process.<sup>2</sup> The most likely

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Contribution from the Research Laboratories of Merck & Co., Inc.

<sup>2</sup> This problem was undertaken at the request of Dr. Charles N. Frey, Director of Scientific Relations, Standard Brands Inc. Results reported herein were presented in evidence by one of the authors (C. R.) before the F.D.A. Bread Standards Hearings in Washington, D. C. on July 25, 1949.

decomposition product is potassium iodide, although other possible reactions may occur, such as iodination of fat, protein, and starch, or even volatilization of iodine.

Standard analytical procedures are complicated by the fact that only  $\approx 1.5$  mg. of iodate is normally employed per lb. of bread. The determination of 0.8–0.9 mg. of iodine in  $\approx 450$  g. of foreign organic material and salts is, at best, uncertain. In this case, the difficulty was magnified by the necessity for identifying in an unequivocal manner the nature of the iodine bearing components. Actual iodimetric analyses (7) of extracts from experimental loaves baked with potassium iodate failed to reveal the presence of residual iodate (cf. also ref. 11). The possibility existed, however, that residual iodate could be reduced during the extraction process prior to analysis. Thus, analyses of extracts from loaves baked without iodate but to which had been added known amounts of potassium iodide and iodate, failed to yield even approximately correct results because of uncertain and shifting endpoints. A further complication was the possibility of transformation of iodine-containing compounds in an extract by microorganisms introduced from the air, which would vitiate the interpretation of analytical data pertaining to such minute amounts of iodine. Finally, the presence of bromate in the flour or the yeast food would interfere with the determination of iodate. For these reasons, standard analytical procedures were abandoned in the solution of this problem.

An unequivocal solution to the problem, and one which would greatly simplify the analytical difficulties, is possible by the use of potassium iodate prepared from radioactive iodine ( $I^{131}$ ; half-life 8.0 days; designated below as  $I$ ). By marking in so distinctive a fashion the iodine originally added to bread dough, the decomposition products of the iodate could be located and determined unquestionably by means of Geiger counter equipment for measuring the electron emission intensity of the  $I^{131}$ . Possible side reactions occurring during the extraction process and in the extraction medium, which might be very significant percentage-wise when minute quantities of iodine are involved, could be minimized or swamped out by the addition of large excesses of ordinary, non-radioactive, isotopic forms of salts such as iodide and iodate. Since the presence of stable isotopic forms does not affect the activity of the minute amounts of radioactive compounds, except for self-absorption effects for which corrections can be made, the stable forms serve as carriers or protectors for the radioactive compounds. Furthermore, because the total weight of material is so greatly increased by the addition of carriers, subsequent handling becomes enormously simplified. By this carrier technique, it was



actually possible to separate the iodide and iodate fractions and to determine the radioactivity of each independently.

### Materials and Methods

**Radioactive Salts.** Radioactive potassium iodide and potassium iodate were obtained from Tracerlab,<sup>3</sup> Inc., Boston, Massachusetts. Purity was investigated first by iodimetric titration (7). In both cases, iodine in the form of iodate and iodide was determined. No iodide was detected in the  $KIO_3$  samples nor was iodate found in the  $KI$  preparations. Furthermore, emission spectrographic analysis revealed only traces of other metallic contaminants. The  $I^{131}$  received from Oak Ridge was tested for isotopic purity by Tracerlab, Inc.

**Baking Experiments.** The basic formula and procedure employed in baking the radioactive loaves were as follows:

| <i>Ingredients and Procedure</i> | <i>Sponge</i> | <i>Dough</i> |
|----------------------------------|---------------|--------------|
| Enriched flour                   | 240.0 g.      | 160 g.       |
| Yeast                            | 8.5 g.        | —            |
| Fermaloid (Special)              | 2.0 g.        | —            |
| Water                            | 134 ml.       | 100 ml.      |
| Salt                             | —             | 8 g.         |
| Shortening                       | —             | 8 g.         |
| Nonfat dry milk solids           | —             | 12 g.        |
| Fermentation time                | 3.5 hr.       | 1.6–2.0 hr.  |
| Fermentation temperature         | 80°F.         | 95°F.        |

Six loaves, to be referred to as A, B, C, D, N, and O, were baked with radioactive potassium iodate. Bakes A and B were preliminary experiments designed primarily to test the experimental technique and to determine whether significant amounts of volatile iodine-containing products were liberated. These loaves were baked in an electric oven, off-gases being aspirated through two scrubbers in series containing successively 10% aqueous sodium hydroxide and 3% alcoholic silver nitrate. These bakes were of somewhat inferior quality probably due to the lower temperature ( $\approx 392^\circ$ – $395^\circ\text{F.}$ ) and longer time (40–45 minutes) required for baking. Two additional loaves, C and D, were baked in a gas oven at  $410^\circ\text{F.}$  for 30 minutes. These loaves were in every respect normal in appearance and quality. Loaves N and O were also baked for 30 minutes in a gas oven, but at a temperature of  $425^\circ\text{F.}$  These loaves were also normal in appearance and quality.

All six radioactive loaves were baked with "Fermaloid" yeast food prepared<sup>4</sup> without potassium iodate, the latter deficiency being supplied as radioactive iodate prior to sponge preparation. About 2 mg. of  $KIO_3$  was used per bake. Each radioactive loaf baked was accom-

<sup>3</sup> The iodine 131 used in these experiments was allocated by the Isotopes Division, U. S. Atomic Energy Commission.

<sup>4</sup> Courtesy of Mr. W. E. Maynard of the Fleischmann Research Laboratories.

panied by a control loaf prepared from normal "Fermaloid." Radioautographs of slices from three loaves (A, N, and O) were taken by placing these slices directly on Eastman "No-Screen" or Type K X-ray film. Fig. 1 shows the radioautographs of a center and an end slice from loaf O placed on No-Screen film two days after baking, and the film developed after an exposure of 14 days. These radioautographs show a great deal of the crumb structure because the bread slices were subjected to slight pressure during the entire period of film exposure to insure close contact with the film.

*Bread Extraction Procedure.* One-quarter loaf of baked bread A and B was dispersed in ammoniacal methanol (10 ml. of concentrated

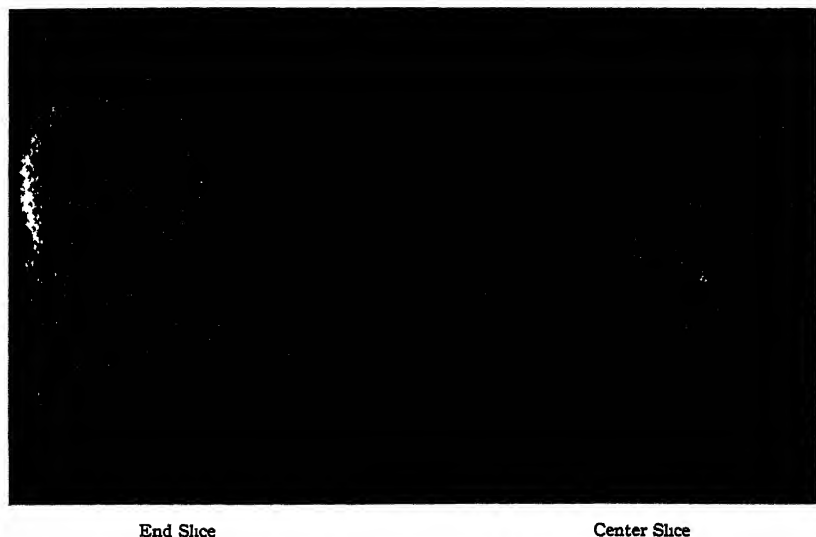


FIG. 1. Radioautographs from loaf O (14 days exposure; "No-Screen" X-ray film).

ammonium hydroxide per 500 ml. of methanol) in a Waring Blendor. The supernatant liquid was filtered through cotton wool, and the residue reextracted several times with additional portions of ammoniacal methanol. This solvent was chosen in order to minimize the amount of extraneous organic material extracted with the soluble salts. Ammonia was added to prevent interaction between iodide and iodate ions, or iodate reduction in general, which occurs in acid solution. Preliminary tests showed that this solvent was adequate for dissolving the small amounts of iodine salts involved.

In the case of C and D, a half-loaf of each was extracted, the volumes of extracting solvent being proportionately larger. Furthermore, an excess of inactive reagent grade potassium iodide and potas-

sium iodate was added to the Waring Blendor before the extractions were performed. Any possibility of extraneous reaction was thus minimized as far as percentage utilization of radioactive iodine is concerned. The extraction of any adsorbed iodine salts was also facilitated.

One-quarter each of loaves N and O were extracted. Thus the initial methanolic extractant (No. 1; added portion-wise) contained the carrier salts and 20 ml. of water to insure their solution, in addition to the standard 10 ml. ammonium hydroxide; extractant No. 2 was similarly composed except for the omission of salts; and extractant No. 3 was simply the standard ammoniacal methanol solution used for

TABLE I  
SUMMARY OF BAKING AND EXTRACTION DATA FOR LOAVES A-D

| Baking Data                          | Loaf No |       |                  |                  |
|--------------------------------------|---------|-------|------------------|------------------|
|                                      | A       | B     | C                | D                |
| Potassium iodate added, mg.          | 1.967   | 1.985 | 2.364            | 2.086            |
| Dough fermentation, proof time, min. | 68      | 70    | 75               | 75               |
| Total weight of dough, g.            | 688     | 690   | 679              | 683              |
| Dough weight per loaf, g.            | 500     | 500   | 500              | 500              |
| Baking temp., °F.                    | 392     | 395   | 410              | 410              |
| Time, min.                           | 45      | 40    | 30               | 30               |
| Weight of loaf, g.                   | 410     | 419   | 436              | 436              |
| Extraction Data                      |         |       |                  |                  |
|                                      | A       | B     | C                | D                |
| Sample of bread, weight g.           | 102.5   | 105.0 | 218.0            | 218.0            |
| Volume of extractant (ml.), 1st      | 400     | 250   | 325 <sup>1</sup> | 360 <sup>1</sup> |
| 2nd                                  | 100     | 100   | 200              | 200              |
| 3rd                                  | 50      | 100   | 150              | 200              |
| 4th                                  | 50      | 50    | 100              | 200              |
| 5th                                  | 50      | 50    | 100              | 100              |
| Total                                | 650     | 550   | 875              | 1060             |
| Volume extract recovered, ml.        | 499     | 420   | 750              | 852              |
| Weight of dried residue, g.          | 67.3    | 70.2  | 134.7            | 129.8            |

<sup>1</sup> Contains 10 ml. water with approximately 100 mg. KI and 100 mg. KIO<sub>3</sub>.

loaves A through D. In these cases, the entire amount of inert carrier iodide and iodate was not supplied as a single addition of solid salt to the Waring Blendor (as in loaves C and D), but the carrier salt mixture was dissolved in the ammoniacal methanol with which the portionwise extraction of the bread was begun. Details relating to all bakings and extractions performed are summarized in Tables I and II.

*Separation of Free Iodide and Iodate in Bread Extracts and Dried Residues.* The determination of the small amounts of iodide and iodate present simultaneously in bread extracts was effected by converting these salts into two silver iodide precipitates, one representing the iodide and a second corresponding to the iodate fraction. These

precipitates were dissolved in aqueous sodium cyanide, and the radioactivity of the solutions measured. Knowing the specific activity (i.e., radioactivity per unit weight) and quantity of the iodate added, the percentage of initial iodine in the several fractions could be readily computed.

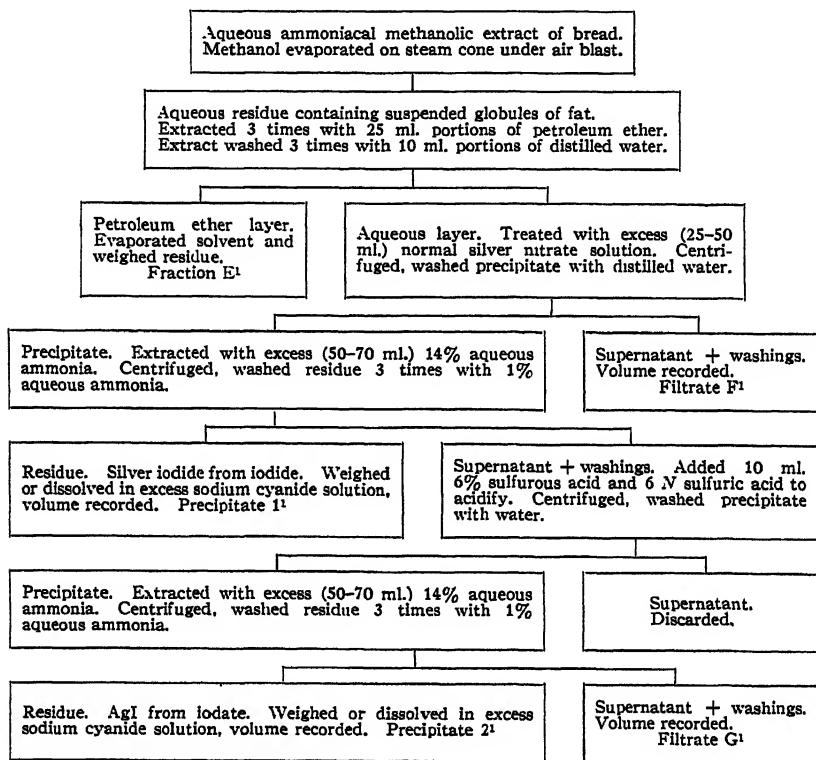
The separation of iodide and iodate is based upon the difference in the solubility of the respective silver salts in aqueous ammonia solution, the iodide being substantially insoluble (about 50 mg. per l.),

TABLE II  
SUMMARY OF BAKING AND EXTRACTION DATA FOR LOAVES N AND O

| Baking Data                             | Loaf No. |        |
|-----------------------------------------|----------|--------|
|                                         | N        | O      |
| Radioactive potassium iodate added, mg. | 2.068    | 2.068  |
| Dough fermentation, proof time, min.    | 60       | 60     |
| Total weight of dough, g.               | 677      | 680    |
| Dough weight per loaf, g.               | 500      | 500    |
| Baking temp., °F.                       | 425      | 425    |
| Time, min.                              | 30       | 30     |
| Weight of loaf, g.                      | 409      | 400    |
| Extraction Data                         |          |        |
|                                         |          |        |
| Sample of bread, weight g.              | 102.25   | 100.00 |
| Approximate number of extractions       |          |        |
| No. 1 extractant, No. extractions       | 3        | 5      |
| 10 ml. ammonium hydroxide               |          |        |
| 20 ml. water                            |          |        |
| potassium iodate, mg.                   | 100.6    | 100.6  |
| potassium iodide, mg.                   | 101.2    | 102.2  |
| to 500 ml. with methanol                |          |        |
| No. 2 extractant, No. extractions       | 3        | 5      |
| 10 ml. ammonium hydroxide               |          |        |
| 20 ml. water                            |          |        |
| to 500 ml. with methanol                |          |        |
| No. 3 extractant, No. extractions       | 3        | 5      |
| 10 ml. ammonium hydroxide               |          |        |
| to 500 ml. with methanol                |          |        |
| Total volume of extractant, ml.         | 1500     | 1500   |
| Total volume of extract, ml.            | 1250     | 1270   |
| Weight of dried residue, g.             | 71.0     | 70.5   |

whereas the iodate is completely soluble. In order to insure complete recovery of the small amounts of radioactive iodide and iodate which might be present, weighed large amounts of reagent grade potassium iodide and iodate were added as carriers to the extract at the beginning of the separation. The separation which comprised six main steps is described in flow sheet form in Fig. 2. After removal of methanol and extraction of fat (Fraction E), silver salts (including iodide and iodate) were precipitated, and the iodate washed out with ammonia. This left

silver iodide precipitate No. 1. The ammoniacal extract was treated with sulfurous and sulfuric acids to reduce the iodate to iodide, which yielded a second silver iodide precipitate No. 2. Silver iodide precipitates Nos. 1 and 2 could be either collected on tared filters and weighed, or dissolved in sodium cyanide solutions. The second technique was adopted for the bulk of this work because the radioactivity measurements were considerably simplified thereby. The petroleum



<sup>1</sup> Radioactive fractions measured.

FIG. 2. Flow sheet for fractionation of bread extract.

ether solution containing fat Fraction E was evaporated in a tared flask and the residue weighed; the volumes of filtrates F and G were recorded. In separation test experiments E (with  $\text{KIO}_3$ ) and H (with  $\text{KI}$ ), water was used instead of bread extract; therefore certain steps were omitted.

During the extraction of loaves A and B, the carrier quantities of inactive potassium iodide and iodate (about 100 mg. each) were added only after the methanol and fat had been removed, since it was believed

on the basis of the literature (2) (6) and preliminary experiments with macro quantities that iodate would be stable in the bread extract if the pH was above 7. Subsequent separation tests with  $\text{KIO}_3$  revealed, however, that in a methanolic bread extract prior to removal of methanol, reduction to iodide can occur on a micro scale even in the alkaline medium (experiment F). On the other hand, addition of carriers before methanol evaporation (experiment G) permitted satisfactory recovery of the radioactivity as iodate.

Similar experiments (I and R) with radioactive potassium iodide indicated that carriers had to be added to the extracts prior to methanol removal in order to minimize conversion (air oxidation?) of iodide to iodate. In view of the above experience, the inactive salts were added to extracts from loaves C, D, N, and O prior to evaporation of methanol. This precaution, coupled with the earlier addition of carrier salts to the Waring Blendor, ensured that the relative amounts of iodide and iodate left in the bread were maintained during the separation procedures, and that extraction of iodine-containing salts is essentially complete, except for possible coprecipitation effects. Assuming no loss of carriers, the total quantities added in these four experiments were about 200 mg. each of potassium iodide and iodate.

While clean, easily filterable silver iodide precipitates were obtained in runs without bread extract, the precipitates from the bread extracts were invariably contaminated with a mucilaginous material which rendered filtration very difficult. Centrifugation was successful in dealing with this problem and hence in experiments C, D, F, G, I, N, O, and R, the silver iodide<sup>3</sup> samples in the centrifuge tubes were dissolved by addition of a weighed amount (100–200 mg.) of reagent grade sodium cyanide and making up to a given volume (25–100 ml.).

The re-extraction procedure to which residues from loaves A, D, N, and O were subjected again involved ammoniacal methanol as solvent. This was merely to ascertain the completeness of the original extraction. The extract was then separated into a petroleum ether (A) and an aqueous (B) fraction. The residue was further treated with acetone to yield another fat fraction (C) and a completely extracted residue (D). In the case of loaves D, N, and O, the aqueous extracts (B) were radioactive. Accordingly, they were examined for iodide and iodate content as mentioned above (Fig. 2) and were found to contain these salts in about the same proportion as was originally extracted. A flow sheet of the re-extraction procedure is shown in Fig. 3.

*Radioactivity Measurements.* The amount of iodine in the several fractions was determined by the radioactivity of measured portions of

<sup>3</sup> It should be emphasized that the purity of the silver iodides is immaterial, since only the radioactivity of precipitates Nos. 1 and 2 is being determined. It is important only to make certain that the silver iodides are completely recovered, and that contaminating impurities are not radioactive.

each sample. The activity was determined with a bell-type Geiger counter tube equipped with a thin mica window weighing 2.7 mg./cm<sup>2</sup>. Samples were placed in circular stainless steel planchets 2.5 cm. in diameter and 7 mm. in depth. The activities of weighed amounts of dried residues were measured directly. Fat fractions were dissolved in a measured volume of petroleum ether or acetone, and aliquots evaporated by means of an infrared lamp. Aqueous filtrates were similarly evaporated prior to counting, as were aliquots of the sodium cyanide solutions of the silver iodides.

Activities are expressed as counts per minutes (c.p.m.) and are corrected for background and for the decay of radioactive iodine using a half-life of 8.0 days. For each baking experiment or separation test, a zero time was chosen, and all activities were corrected for iodine decay

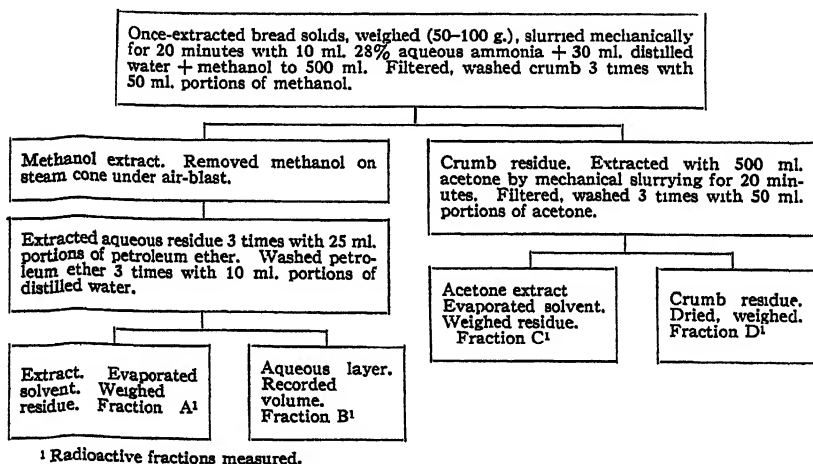


FIG. 3. Flow sheet for re-extraction of bread residue.

back to this starting time. The amount of iodine present in any given fraction could then be expressed as a percentage of the total iodine. No coincidence corrections were made since relatively low activities were measured throughout. During the bulk of this work, a National Bureau of Standards Radium D + E standard preparation was measured daily as a check on sensitivity variations of the counter tube, and appropriate corrections made for daily variations.

Every fraction possessing significant activity was usually measured in triplicate. Furthermore, each measurement involved a different amount of sample, i.e. varying aliquots of solution or several weights of residue. This was done to ascertain whether the residues were thick enough to absorb an appreciable part of the electrons emitted by the radioiodine. In the case of the fat fractions, and of course in the dried,

extracted bread residues, self-absorption (1, 4, 12) of radiation was important, and appropriate correction factors were established experimentally. The above correction data are not reported since they apply strictly to the particular measuring equipment employed.

*Standardizations and Separation Tests.* The radioactivity of the stock  $\text{KIO}_3$  and  $\text{KI}$  salts was determined by weighing several mgm. of salt into a 50–100 ml. volumetric flask, dissolving in water, and measuring the activity of the aliquot residues as described in the preceding section. The specific activities of these salts, expressed as counts per min. per mgm., were calculated for those times taken as the starting points of pertinent separation and baking experiments.

The efficiency of the iodide-iodate separation procedure was tested separately with radioactive potassium iodide and with radioactive

TABLE III  
TESTS OF SEPARATION EFFICIENCY

| Expt. | Radioactive-Component | Medium        | Carrier added           | % of Total Measured Activity |        |              |            |            |
|-------|-----------------------|---------------|-------------------------|------------------------------|--------|--------------|------------|------------|
|       |                       |               |                         | Iodide                       | Iodate | Fat Fraction | Filtrate F | Filtrate G |
| E     | Iodate                | Water         | Present                 | 4.4                          | 95.6   | —            | Discarded  | Discarded  |
| F     | Iodate                | Bread Extract | After Methanol Removal  | 87.6                         | 11.2   | 0.4          | 0.3        | 0.3        |
| G     | Iodate                | Bread Extract | Before Methanol Removal | 7.3                          | 92.4   | 0.3          | Discarded  | Discarded  |
| H     | Iodide                | Water         | Present                 | 99.3                         | 0.7    | —            | Discarded  | Discarded  |
| I     | Iodide                | Bread Extract | After Methanol Removal  | 89.7                         | 9.9    | 0.4          | Discarded  | Discarded  |
| R     | Iodide                | Bread Extract | Before Methanol Removal | 97.0                         | 2.4    | 0.1          | 0.4        | 0.1        |

potassium iodate added to water and to ammoniacal methanol extracts of bread. Results are reported in Table III which gives the activities of the various fractions in per cent of total activity. The theoretical activity can be calculated for any experiment from the weight of radioactive salt employed and its specific activity. Because of the difficulties inevitably attending these radioactivity measurements, and because of the large number of different samples measured, the measured total activity may deviate significantly from the theoretical activity. Whenever this difference exceeded 1% in these separation tests and in the baking experiments reported in the next Section, the per cent of total activity in the several fractions was recomputed on the basis of the measured total activity as 100%.



This permits a more convenient comparison of the distribution of activity among the different fractions of a given experiment.

Three experiments<sup>6</sup> were performed with the iodate. Experiment E was carried out in water in the presence of added inert iodide and iodate as a preliminary trial of the validity of the separation. Apparently a small amount (4.4%) of the initial active potassium iodate is retained by the iodide fraction. In this test the silver iodide precipitates from the iodide and iodate fractions were filtered and weighed prior to solution in cyanide for determination of radioactivity. The weights of both precipitates were about 99% of theoretical.

Experiment F is the counterpart of E made on a bread extract. Excess salts were not added, however, until after evaporation of the methanol. No attempt was made to weigh the silver iodide precipitates prior to solution in cyanide. It is obvious that considerable reduction ( $\approx 88\%$ ) of the minute amount of iodate to iodide occurred during the removal of the methanol from the extract.

In experiment G, also performed with an ammoniacal methanol bread extract, the carrier salts were added before evaporation of methanol in order to minimize the extent of possible iodate reduction. The effectiveness of this swamping procedure is demonstrated by the fact that only about 7% of the radioactive iodine, introduced originally as iodate, was present in the iodide fraction. This may be due more to coprecipitation effects enhanced by the presence of protein, starch, and salts in the extract than to actual reduction of iodate by bread components.

Experiments<sup>7</sup> H, I, and R were performed with radioactive potassium iodide. In water (experiment H) the separation is virtually quantitative; and the weights of silver iodide precipitates Nos. 1 and 2 prior to solution in cyanide agree with the expected weights. In the presence of bread extract, however, a small amount of iodide is retained by the iodate fraction. Thus, in experiment I in which the inert carriers were added after removal of methanol, this amounts to  $\approx 10\%$  and may represent a small degree of (air ?) oxidation of iodide to iodate in basic solution. This effect is practically eliminated, however, by supplying the carrier salts at the very start of the separation procedure, i.e. before evaporation of the alcohol. Thus experiment R shows that only 2.4% of the total activity resides in the iodate fraction. The protecting effect of the carrier in minimizing the extent of undesirable reactions of the small amounts of active material present, until a final separation can be achieved, is thus again demonstrated.

The above series of separation tests shows the necessity for addition of protective carrier salts at the very start of the separation procedure.

<sup>6</sup>  $\approx 100$  mg. of each carrier salt used in these experiments.

<sup>7</sup> 100 mg. of each carrier used in experiments H and I; 200 mg. of each used in experiment R.

Although the separations of the iodide and iodate fractions are not clean from a quantitative point of view, the extent of contamination of iodide by iodate and vice versa has been evaluated in the separation tests. This will in no way invalidate the general conclusions as to the course of the iodate decomposition, but will tend to yield somewhat high iodate values.

### Results

The distribution of radioactivity among the several extraction fractions and residues from the six loaves of bread baked with radioactive potassium iodate is reported in Tables IV-VI. Successive columns represent the number and nature of the fraction involved, the radioactivity in c.p.m., the per cent of theoretical total activity of the

TABLE IV  
PRELIMINARY BAKING EXPERIMENTS A AND B; ELECTRIC OVEN

| Fraction                |                   | Experiment A <sup>1</sup> |                           |                              | Experiment B <sup>2</sup> |                                        |
|-------------------------|-------------------|---------------------------|---------------------------|------------------------------|---------------------------|----------------------------------------|
| Number                  | Nature            | c.p.m.                    | % of Theoretical Activity | % of Measured Total Activity | c.p.m.                    | % of Theoretical Activity <sup>4</sup> |
| —                       | Off-gas           | 0                         | 0                         | 0                            | 100 (?)                   | 0.02 (?)                               |
| 1                       | Iodide            | 330,500                   | 59.7                      | 65.4                         | 176,000                   | 40.9                                   |
| 2                       | Iodate            | 23,300 <sup>3</sup>       | 4.2                       | 4.6                          | 63,640                    | 14.8                                   |
| E                       | Fat Fraction      | 2,610                     | 0.5                       | 0.5                          | 3,730                     | 0.9                                    |
| F                       | Filtrate          | 570                       | 0.1                       | 0.1                          | 800                       | 0.2                                    |
| G                       | Filtrate          | 980                       | 0.2                       | 0.2                          | 570                       | 0.1                                    |
| —                       | Extracted Residue | 147,000                   | 26.6                      | 29.1                         | 116,700                   | 27.2                                   |
| A                       | Fat (Pet. ether)  | 5,150                     | (0.9)                     | (1.0)                        | —                         | —                                      |
| B                       | Aqueous Fraction  | 990                       | (0.2)                     | (0.2)                        | —                         | —                                      |
| C                       | Fat (Acetone)     | 10,300                    | (1.9)                     | (2.1)                        | —                         | —                                      |
| Total Measured Activity |                   | 504,960                   | 91.3                      | 100                          | 361,440                   | 84.1                                   |
| Theoretical Activity    |                   | 553,000                   | 100                       | 109.5                        | 430,000                   | 100                                    |
| Deficiency              |                   | 98,040                    | 8.7                       | —                            | 68,560                    | 15.9                                   |

<sup>1</sup> Calculated initial weight  $KIO_3$  in bread sample 0.358 mg. Carriers added after removal of methanol (100.3 mg. iodate; 100.8 mg. iodide).

<sup>2</sup> Corrected for loss of precipitate No. 2 as described in test.

<sup>3</sup> Calculated initial weight  $KIO_3$  in bread sample 0.360 mg. Carriers added after removal of methanol (100.0 mg. iodate; 100.3 mg. iodide).

<sup>4</sup> % of measured total activity not computed because this activity balance sheet is obviously deficient.

bread sample, and the per cent of the measured total activity present in a given fraction, recalculated as described in the preceding section. Figures in parentheses are not included in the totals.

Table IV summarizes results obtained with the loaves (experiments A and B) baked in an electric oven. These preliminary experiments are atypical because of the non-uniform heat and lower temperatures

prevailing in the oven employed for baking. Furthermore, in the extraction of these loaves, carrier salts were not added until after methanol was removed from the bread extract, so that the values of iodate and iodide activities are in doubt. In both cases an attempt was made to collect and weigh the silver iodide precipitates prior to solution in cyanide for activity determination. In contrast to the virtually complete recoveries obtained from water solutions (experiments E and H), silver iodide precipitate weights tended to run con-

TABLE V  
BAKING EXPERIMENTS C AND D; GAS OVEN

| Fraction                |                                   | Experiment C <sup>1</sup> |                           |                              | Experiment D <sup>1</sup> |                           |                              |
|-------------------------|-----------------------------------|---------------------------|---------------------------|------------------------------|---------------------------|---------------------------|------------------------------|
|                         |                                   | c.p.m.                    | % of Theoretical Activity | % of Measured Total Activity | c.p.m.                    | % of Theoretical Activity | % of Measured Total Activity |
| Number                  | Nature                            |                           |                           |                              |                           |                           |                              |
| 1                       | Iodide                            | 39,050                    | 71.7                      | 72.8                         | (34,800)                  | (72.8)                    | (75.1)                       |
| 2                       | Iodate                            | 1,659                     | 3.0                       | 3.0                          | (1,650)                   | (3.4)                     | (3.6)                        |
| E                       | Fat Fraction                      | 20 (?)                    | 0.04 (?)                  | 0.04 (?)                     | 0                         | 0                         | 0                            |
| F                       | Filtrate                          | 100 (?)                   | 0.2 (?)                   | 0.2 (?)                      | 0                         | 0                         | 0                            |
| G                       | Filtrate                          | 20 (?)                    | 0.04 (?)                  | 0.04 (?)                     | 0                         | 0                         | 0                            |
| —                       | Extracted Residue                 | 12,900                    | 23.6                      | 23.9                         | —                         | —                         | —                            |
| A                       | Fat. (Pet. Ether)                 | —                         | —                         | —                            | 0                         | 0                         | 0                            |
| C                       | Fat (Acetone)                     | —                         | —                         | —                            | 200 (?)                   | 0.4 (?)                   | 0.4 (?)                      |
| D                       | Re-extracted <sup>2</sup> Residue | —                         | —                         | —                            | 0                         | 0                         | 0                            |
| 1                       | Iodide (2nd Extraction)           | —                         | —                         | —                            | (9,100)                   | —                         | —                            |
| 2                       | Iodate (2nd Extraction)           | —                         | —                         | —                            | (600)                     | —                         | —                            |
| —                       | Total Iodide                      | —                         | —                         | —                            | 43,900                    | 91.8                      | 94.8                         |
| —                       | Total Iodate                      | —                         | —                         | —                            | 2,250                     | 4.7                       | 4.8                          |
| Total Measured Activity |                                   | 53,750                    | 98.6                      | 100                          | 46,350                    | 96.8                      | 100                          |
| Theoretical Activity    |                                   | 54,500                    | 100                       | 101.4                        | 47,870                    | 100                       | 103.3                        |
| Deficiency              |                                   | 750                       | 1.4                       | —                            | 1,520                     | 3.2                       | —                            |

<sup>1</sup> Calculated initial weight  $KIO_3$  in bread sample 0.870 mg. Carriers added at time of extraction ( $\approx 100$  mg. each) and before methanol evaporation ( $\approx 100$  mg. each).

<sup>2</sup> Calculated initial weight  $KIO_3$  in bread sample 0.764 mg. Carriers added at time of extraction ( $\approx 100$  mg. each) and prior to methanol evaporation ( $\approx 100$  mg. each).

<sup>3</sup> Re-extraction in the presence of  $\approx 50$  mg. of iodate and  $\approx 50$  mg. iodide.

siderably higher than theoretical due to the presence of mucilaginous contaminants which adhered to the precipitates. The single exception occurred in experiment A where, due to peptization, the recovery of the precipitate No. 2 corresponding to the iodate fraction was only 76.5%. The c.p.m. value listed for this fraction in Table IV is corrected for this loss.

The tendency of the ammonia-extracted silver iodide residues from bread extract to peptize when washed was always extremely troublesome, and in some cases the iodides could not be centrifuged after

washing unless some sodium nitrate was added to the supernatant as coagulant. The peptization was caused exclusively by bread colloids, since it was never observed in silver iodide residues from pure water solutions.

An attempt to re-extract the residue from experiment A with ammoniacal methanol yielded but little additional activity. It was found subsequently that extraction efficiency was increased by the presence of carriers, and that re-extraction by a slurring procedure was more effective. The recovery of silver iodide precipitates by

TABLE VI  
BAKING EXPERIMENTS N AND O; GAS OVEN

| Fraction                     |                                   | Experiment N <sup>1</sup> |                           | Experiment O <sup>3</sup> |                           |
|------------------------------|-----------------------------------|---------------------------|---------------------------|---------------------------|---------------------------|
| Number                       | Nature                            | c.p.m.                    | % of Theoretical Activity | c.p.m.                    | % of Theoretical Activity |
| 1                            | Iodide                            | (145,200)                 | (87.5)                    | (141,600)                 | (85.7)                    |
| 2                            | Iodate                            | (4,340)                   | (2.6)                     | (12,400)                  | (7.5)                     |
| E                            | Fat Fraction                      | ≈ 1,400                   | 0.8                       | 125                       | 0.1                       |
| F                            | Filtrate                          | ≈ 5,860                   | 3.5                       | ≈ 700                     | 0.4                       |
| G                            | Filtrate                          | 930                       | 0.5                       | 730                       | 0.4                       |
| A                            | Fat (Pet. Ether)                  | ≈ 50                      | 0                         | —                         | —                         |
| D                            | Re-extracted Residue <sup>2</sup> | ≈ 5,400                   | 3.3                       | 6,700                     | 4.1                       |
| 1                            | Iodide (2nd Extraction)           | (3,540)                   | —                         | (2,920)                   | —                         |
| 2                            | Iodate (2nd Extraction)           | (≈ 130)                   | —                         | (100)                     | —                         |
| —                            | Total Iodide                      | 148,740                   | 89.5                      | 144,520                   | 87.4                      |
| —                            | Total Iodate                      | 4,470                     | 2.7                       | 12,500                    | 7.5                       |
| Total Measured Activity      |                                   | 166,850                   | 100.4                     | 165,275                   | 99.9                      |
| Theoretical Activity         |                                   | 166,200                   | 100                       | 165,300                   | 100                       |
| Excess (N) or Deficiency (O) |                                   | 650                       | 0.4                       | 25                        | 0.1                       |

<sup>1</sup> Calculated initial weight  $KIO_3$  in bread sample 0.382 mg. Carriers added at time of extraction (≈100 mg. each) and before evaporation of methanol (≈100 mg. each).

<sup>2</sup> Re-extraction in the presence of ≈50 mg. iodate and iodide.

<sup>3</sup> Calculated initial weight  $KIO_3$  in bread sample 0.380 mg. Carriers added at time of extraction (≈100 mg. each) and before evaporation of methanol (≈100 mg. each).

filtration appears to be of doubtful value since, in both experiments A and B, large activity deficiencies (differences of 9 and 16% between theoretical and total measured activities) were noted. Accordingly, in subsequent experiments the filtration practice was discontinued, and all washings were performed in centrifuge tubes. The justification for this step is seen from the improved activity balance sheets characterizing the remaining experiments. It is obvious, however, even from these preliminary experiments, that a negligible amount of iodine is liberated among the gaseous products, and that the reduction of iodate during baking is extensive.

Tables V and VI summarize the results obtained with the four loaves (experiments C, D, N, and O) baked in a gas oven. All four cases illustrate the increased initial extraction efficiency in the presence of carriers, which were added before evaporation of methanol. In the last three experiments, the once-extracted residues were re-extracted by a slurring technique in the presence of carrier. By this device, additional iodate and iodide were obtained, in about the same ratio as in the initial extractions. In experiment N, filtrate F exhibited an exceptionally high (3.5%) activity. This is probably due to incomplete precipitation of the iodate and/or iodide. Accordingly, the value of 2.7% reported in this experiment for iodate is probably low, and may actually be as high as 6.2%.

The uniform distribution of the radioactive salts in the loaves and in the samples taken for analysis was demonstrated qualitatively by the radioautographs of end and center slices (Fig. 1). The excellent radioactivity balance sheets obtained with unequivocal bakings C, D, N, and O constitute quantitative evidence of proper sampling.

### Discussion

In all of these experiments, the largest fraction of total activity present as iodate was 14.8%. This occurred in the preliminary experiment B, which was atypical because of unrepresentative baking conditions and was suspected on the grounds of incomplete separation and because of a very incomplete activity balance. In the remaining baking experiments, which included the four unequivocal bakings C, D, N, and O, the amount of iodine found as unconverted iodate was considerably less, i.e. 4.6, 3.0, 4.8, 6.2, and 7.5%. This leads to the conclusion that less than 7.5% of the original iodate is left in this form after the baking process. Even this value is undoubtedly high because the separation of iodide from iodate was not quantitative (see section on separation tests). The retention of iodate by iodide would not affect the results appreciably because of the small amount of iodate involved. Since the bulk of the radioactivity is present as iodide, however, the presence of several per cent of iodide in the iodate fraction (cf. experiment R) would lead to significantly higher apparent values for iodate.

It appears likewise that the principal iodine-containing product formed is potassium iodide. One might be inclined to deduce from experiments A, B, and C (Tables IV and V) that only about half or two-thirds of the original iodine is converted to iodide, and that a considerable fraction (24-27%) is permanently fixed in the dried residue. However, experiments D, N, and O (Tables V and VI), in which more efficient extractions were performed, show quite clearly

that the activity of the once extracted bread is not permanently bound but, on the contrary, extractable; actually about 90% of the initial iodate is transformed to iodide. Although the small amount of activity (3-4%) left in the re-extracted residues (experiments N and O) may actually be retained as combined iodine, it is more likely present simply as adsorbed salt, hence completely extractable as was found in experiment D. No radioactivity was observed in the scrubbed off-gases; and the activities of the fat fractions were small and variable, and probably were due to slight contamination by the active aqueous phases extracted. It is evident from the above experiments that potassium iodate is extensively decomposed to potassium iodide during the baking of bread.

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## BAKING PROPERTIES AND PALATABILITY STUDIES OF SOY FLOUR IN BLENDS WITH HARD WINTER WHEAT FLOUR<sup>1</sup>

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### ABSTRACT

A commercially milled and bleached hard red winter wheat flour containing 13.9% protein was baked with 4% and 8% of a commercial hexane-extracted, a commercial full-fat, and an ethanol extracted soy flour. Excellent bread was made with blends containing up to 8% soy flour with the hard red winter wheat flour, the quality of the bread as measured by crumb grain and loaf volume being equal to that for wheat flour alone provided the quantity of potassium bromate used in the baking formula was increased. The average increase in baking absorption for each 1% of soy flour added was over 1%. Crumb colors for bread containing soy flour were creamy-gray, the degree of which was dependent on the kind and amount of soy flour used. Soy flours have specific baking properties and potentialities in blends with hard red winter wheat flour. These potentialities are not expressed, however, unless suitable alterations are made in the baking method.

For palatability studies, bread was made from wheat flour alone and from wheat-soy flour blends containing 4% and 8% of two commercial hexane-extracted, two commercial full-fat, and two ethanol-extracted soy flours. These breads were scored for tenderness, soylike flavor, desirability of flavor, and acceptability of bread. Statistical analysis of the data obtained in this study indicated that bread made from wheat flour alone was preferred to that made from soy-wheat flour blends. In addition, one of the hexane soy flours was more easily detected than the other at the 8% level; 8% of soy flour was more easily detected than 4%; and ethanol-extracted soy flours were preferred to full-fat flours at the 8% level.

The addition of 1 mg. of potassium bromate per 100 g. of wheat flour by Bohn and Favor (2) improved loaf volume and internal characteristics of the bread made from wheat-soy flour blends. Studies carried out in the Soft Wheat Quality Laboratory at Wooster, Ohio (3) showed that bread of excellent loaf volume and crumb grain could be made from blends containing up to 8% soy flour with hard red spring wheat flour, providing the quantity of potassium bromate used in the baking formula was increased with the amount of soy flour used.

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Bayfield and Swanson (1) state that it was necessary to increase potassium bromate with increasing percentages of soy flour, and that when proper adjustments in formula and procedure were made, bread containing as much as 6% soy flour was equal to the control, except for crumb color. These breads, however, were not judged for palatability. Accordingly, the cooperation of the Bureau of Human Nutrition and Home Economics was requested to study the acceptability of such breads.

A hard red *winter* wheat flour was used for the palatability studies and they in turn were expanded sufficiently to obtain data comparable to those reported by Finney (3), in previous studies with soy flour in blends with a hard red *spring* wheat flour. In this way a comparison was made of the baking properties of the two classes of hard wheat flour, winter and spring, when in blends with soy flour. This paper describes the baking properties and behavior of blends containing 4% and 8% soy flour with hard red winter wheat flour containing 13.9% protein, and outlines the judging procedure employed and gives a statistical summary of data obtained in making the acceptability tests.

### Materials and Methods

Two commercial hexane-extracted (A and B), two commercial full-fat (C and D), and two ethanol-extracted (E and F) soy flours prepared by the Northern Regional Research Laboratory were used in the breads made for palatability tests. Three composite soy flours (A + B, C + D, and E + F), however, were used in the studies concerned with evaluating the bread baking properties of the three types of soy flour. These three composites of soy flour contained 43.0%, 31.9% and 46.6% protein, respectively. Four and 8% of each of these three composite soy flours were blended with a commercially milled and bleached hard red winter wheat flour containing 13.9% protein. Each of these blends (100 g. wheat flour plus soy flour) was baked with 0, 3, 4, and 5 mg. of potassium bromate per 100 g. flour and with 4% nonfat milk solids in the formula. Wheat flour alone was baked into bread with 0, 2, 3, and 4 mg. of bromate and 4% nonfat milk solids, and with 0, 1, 2, and 3 mg. of bromate when the milk solids were omitted. Additional ingredients used in all bakes were 6 g. sugar, 1.5 g. salt, 3 g. shortening, 2 g. yeast, 0.25 g. malt syrup (120°L), and water as needed. An optimum mixing time with the straight dough procedure and a 3-hr. fermentation at 30°C. were employed. All punching and panning were performed mechanically. Baking time was 25 min. at 221°C. Bakings were replicated at least twice. A third replicate was made when loaf volumes differed by more than 25 cc. Data for all replicates were averaged.



Each series of samples for palatability tests included six loaves containing 4% and 8% of three of the six soy flours, two loaves duplicating one of the soy flours at each of the two percentage levels, and one check loaf of wheat flour only. Twelve replications of this nine loaf series were baked in the Federal Soft Wheat Laboratory at Wooster, Ohio, using the above formula together with 4% nontat milk solids and optimum bromate. Immediately after cooling, each nine-loaf series was sent by air to the laboratory at Beltsville for the palatability tests. The elapsed time between baking and the palatability tests was approximately 48 hr.

The palatability tests were preceded by four training periods in which judges were taught to recognize soy flavor, to judge tenderness, and to score these two factors quantitatively. On another part of the score card they were asked to state preferences for samples in terms of the desirability of flavor and general acceptability of the bread. Thus, information on amount of soy flavor detected in breads containing the various kinds of flours was recorded independent of whether or not the flavor was enjoyed.

The samples presented at each of 12 judging sessions included coded slices of bread containing no soy flour and 4% and 8% of one hexane-extracted, one full-fat, and one ethanol-extracted soy flour. Labeled slices of bread made from wheat flour alone were included for the purpose of comparison. The judging room was partially darkened to minimize color and texture differences. Cool water and slices of apple were allowed as needed to clear the mouth of bread flavors.

The presentation of samples during the 12 bread-judging periods was such that each judge made eight scorings for tenderness, soylike flavor, desirability of flavor, and acceptability of bread for each of the six soy flours at each per cent level and 12 scorings for wheat flour alone. Oily, musty, rancid, sour, and bitter were adjectives listed for describing bread flavors. Tenderness was scored from five (normal) to three (very tender or very tough), soylike flavor from five (none) to one (very strong), and desirability of flavor and acceptability of bread from five (very good) to one (not acceptable). Student's "t" test was used to test for significance between various combinations of mean scores.

### Results

The baking absorptions, mixing times, potassium bromate requirements, and bread-crumbs color scores for the 13.9% protein hard winter wheat flour alone and when blended with 4% and 8% of the three different soy flours are given in Table I, and the loaf volumes and crumb grain scores are shown graphically in relation to the potassium

TABLE I

ABSORPTIONS, MIXING TIMES, and POTASSIUM BROMATE REQUIREMENTS FOR HARD RED WINTER WHEAT FLOUR DOUGHS CONTAINING 0%, 4% AND 8% OF 3 DIFFERENT SOY FLOURS TOGETHER WITH THE CRUMB COLOR SCORES FOR BREAD BAKED THEREFROM

| Kind and Per Cent of Soy-Flour<br>in Blends | Baking <sup>1</sup><br>Absorption | Mixing<br>Time  | Potassium<br>Bromate<br>Requirement | Optimum<br>Bread-<br>Crumb<br>Color <sup>2</sup> |
|---------------------------------------------|-----------------------------------|-----------------|-------------------------------------|--------------------------------------------------|
|                                             | %                                 | min             | mg                                  |                                                  |
| 4% Soy Flour                                |                                   |                 |                                     |                                                  |
| Com'l oil-free A + B <sup>3</sup>           | 74.3                              | 2 $\frac{3}{4}$ | 4                                   | 87                                               |
| Com'l full-fat C + D                        | 73.1                              | 2 $\frac{3}{4}$ | 4                                   | 85                                               |
| Ethanol-extracted E + F                     | 75.9                              | 2 $\frac{3}{4}$ | 4                                   | 86                                               |
| 8% Soy Flour                                |                                   |                 |                                     |                                                  |
| Com'l oil-free A + B                        | 78.4                              | 2 $\frac{3}{4}$ | 5                                   | 81                                               |
| Com'l full-fat C + D                        | 75.8                              | 2 $\frac{3}{4}$ | 5                                   | 73                                               |
| Ethanol-extracted E + F                     | 81.2                              | 2 $\frac{3}{4}$ | 4                                   | 74                                               |
| No Soy Flour                                |                                   |                 |                                     |                                                  |
| Control                                     | 70.1                              | 2 $\frac{3}{4}$ | 3                                   | 103                                              |
| Nonfat M.S. omitted <sup>4</sup>            | 67.6                              | 2 $\frac{3}{4}$ | 1                                   | 107                                              |

<sup>1</sup> 14% moisture. When bromate was omitted in the formula, absorptions were reduced 1%.

<sup>2</sup> Loaves having the optimum crumb grains and loaf volumes, in general, had the best crumb colors.

<sup>3</sup> Equal parts of each at 14% moisture.

<sup>4</sup> All other doughs contained 4% nonfat milk solids.

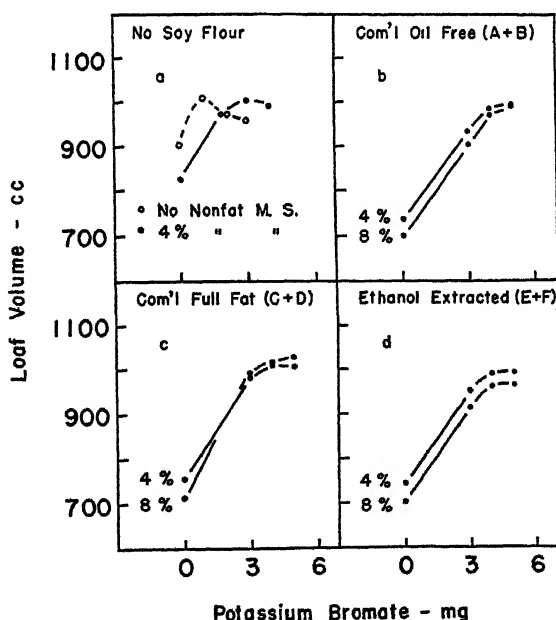


FIG. 1. Loaf volumes for a hard red winter wheat flour (13.9% protein) baked with and without the addition of nonfat milk solids and with 4% and 8% of each of 3 different soy flours at several potassium bromate levels with milk solids in the formula.

bromate requirements in Figs. 1 and 2. The bromate requirement given in Table I is the amount of potassium bromate required to produce approximately the maximum loaf volume shown in Fig. 1.

*Loaf Volume and Bromate Requirement.* The wheat flour without soy flour and without milk solids produced a loaf volume of 910 cc. (Fig. 1a) with no potassium bromate in the formula. The addition of 4% nonfat milk solids, however, reduced loaf volume 80 cc. When the hard winter wheat flour was blended with 4% and 8% of soy flour and baked into bread by the same formula (4% milk solids and no bromate)

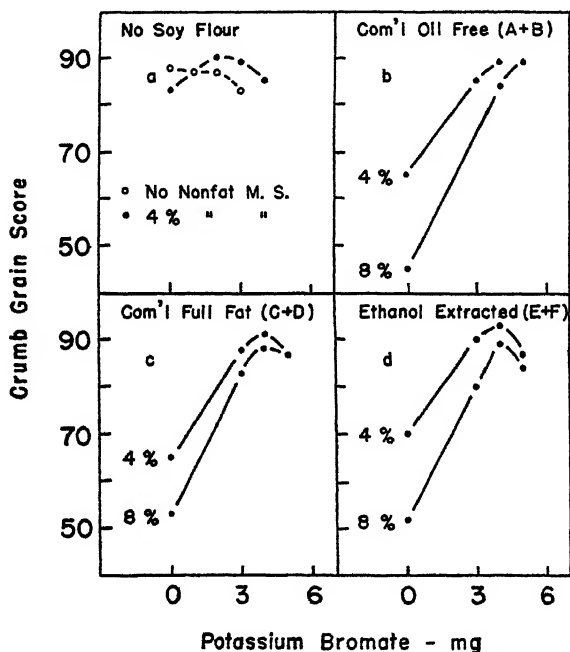


FIG. 2. Crumb grain scores for a hard red winter wheat flour (13.9% protein) baked with and without the addition of nonfat milk solids and with 4% and 8% of each of 3 different soy flours at several potassium bromate levels with milk solids in the formula.

further material reductions in loaf volume (Fig. 1a to 1d) were obtained. For example, 4% and 8% of the commercial oil-free soy flour reduced loaf volume 90 cc. and 127 cc. respectively, below that for milk solids alone. The same concentrations of full-fat and ethanol-extracted soy flours produced equally large reductions. In every instance the reduction in loaf volume was greater for 8% than for 4% soy flour. The optimum loaf volume (Fig. 1a) for the hard winter wheat flour without soy flour and without milk solids was produced with only 1 mg. of potassium bromate; whereas with 4% milk solids 3 mg. were required. When 4% and 8% of each of the three soy

flours were included with milk solids in the formula, 4 to 5 mg. of bromate were required for optimum loaf volume (Fig. 1b, 1c, and 1d), although the commercial full-fat soy flour gave nearly optimum volumes equal to that for milk solids alone with only 3 mg. of bromate. The optimum volumes for both concentrations of all three soy flours were equal to or slightly greater than the optimum for milk solids alone, except the 8% ethanol-extracted soy flour.

*Bread Crumb Grain.* The bread crumb grain data (Fig. 2) are principally of interest from the standpoint of a comparison with loaf volumes obtained at corresponding potassium bromate levels. First it will be noted that additions of each of the soybean flours strikingly decreased the crumb grain scores (Fig. 2b, 2c, and 2d) as compared with the hard winter wheat flour alone (Fig. 2a) when no bromate was used, and that this depressing or reducing effect, also noted for loaf volume, was much greater for 8% than for 4% soy flour. These scores, however, gradually improved as the quantity of bromate increased, in the same manner as was noted for loaf volume, so that the loaf volume and crumb grain curves roughly parallel each other. Most important, the optimum crumb grains and optimum loaf volumes were produced with approximately the same quantity of bromate in the formula. In addition, the optimum crumb grains for the 4% soy flour blends were equal to or slightly better than those obtained for wheat flour alone; whereas those for the 8% soy flour blends were slightly poorer. Thus the effects on crumb grain scores are so similar to those on loaf volume that the latter may for all practical purposes be used alone to evaluate these effects. It should be noted, nevertheless, that the blends with the ethanol-extracted soy flour (Fig. 2d) are more sensitive to the detrimental effects of too much bromate than are the blends of the other two soy flours.

*Bread Crumb Color.* The loaves having optimum crumb color scores (last column of Table I) also had the optimum or nearly optimum loaf volumes and crumb grains. The crumb color value of 103, which characterizes the control, is considered excellent. The crumb colors of 85 to 87 for all 4% soy flour blends would be noticeably creamy or creamy-gray to some consumers of white bread; whereas the bread crumb colors of 73 and 74 for the blends containing 8% of the commercial full-fat and ethanol-extracted soy flour would be noticed by many.

*Baking Absorption and Mixing Time.* The water absorptions (Table I) varied considerably for the three different soy flour composites. For example, it was found necessary to add 1.05, 0.75, and 1.45% additional water for each 1% of the commercial oil-free, commercial full-fat, and ethanol-extracted soy flour, respectively. Mix-

ing requirement of the hard winter wheat flour was not altered by the addition of soy flour.

The mean scores for soylike flavor, desirability of flavor, and acceptability of bread together with the levels of significance for differences between types and percentages of soy flour are given in Tables II, III, and IV.

*Tenderness.* There seemed to be no agreement among judges as to whether samples were more tender or more tough than normal.

TABLE II  
STATISTICAL SIGNIFICANCE OF DIFFERENCES IN STRENGTH OF SOY-LIKE FLAVOR

| Comparisons                                  | Sample and % Level of Soy                                                                        | Mean Score | Mean Score | Sample and % Level of Soy | Significance |
|----------------------------------------------|--------------------------------------------------------------------------------------------------|------------|------------|---------------------------|--------------|
| a) Within type at each level                 | A <sub>4</sub>                                                                                   | 4.04       | 4.00       | B <sub>4</sub>            | —            |
|                                              | A <sub>8</sub>                                                                                   | 3.94       | 3.50       | B <sub>8</sub>            | **           |
|                                              | C <sub>4</sub>                                                                                   | 3.88       | 3.98       | D <sub>4</sub>            | —            |
|                                              | C <sub>8</sub>                                                                                   | 3.47       | 3.64       | D <sub>8</sub>            | —            |
|                                              | E <sub>4</sub>                                                                                   | 4.29       | 4.04       | F <sub>4</sub>            | —            |
|                                              | E <sub>8</sub>                                                                                   | 3.96       | 3.75       | F <sub>8</sub>            | —            |
| b) Between wheat and soy flour at each level | Mean score of wheat (4.72) significantly different from mean score of each of the soy samples.** |            |            |                           |              |
| c) Between levels within a type              | (A+B) <sub>4</sub>                                                                               | 4.02       | 3.94       | A <sub>8</sub>            | —            |
|                                              | (A+B) <sub>8</sub>                                                                               | 4.02       | 3.50       | B <sub>8</sub>            | **           |
|                                              | (C+D) <sub>4</sub>                                                                               | 3.93       | 3.56       | (C+D) <sub>8</sub>        | **           |
|                                              | (E+F) <sub>4</sub>                                                                               | 4.15       | 3.85       | (E+F) <sub>8</sub>        | **           |
|                                              | B <sub>4</sub>                                                                                   | 4.00       | 3.50       | B <sub>8</sub>            | **           |
|                                              |                                                                                                  |            |            |                           |              |
| d) Between types within a level              | (A+B) <sub>4</sub>                                                                               | 4.02       | 3.93       | (C+D) <sub>4</sub>        | —            |
|                                              | (A+B) <sub>8</sub>                                                                               | 4.02       | 4.15       | (E+F) <sub>4</sub>        | —            |
|                                              | (C+D) <sub>4</sub>                                                                               | 3.93       | 4.15       | (E+F) <sub>8</sub>        | —            |
|                                              | (C+D) <sub>8</sub>                                                                               | 3.56       | 3.85       | (E+F) <sub>8</sub>        | *            |
|                                              | (C+D) <sub>8</sub>                                                                               | 3.56       | 3.94       | A <sub>8</sub>            | *            |
|                                              | (C+D) <sub>8</sub>                                                                               | 3.56       | 3.50       | B <sub>8</sub>            | —            |
|                                              | (E+F) <sub>8</sub>                                                                               | 3.85       | 3.94       | A <sub>8</sub>            | —            |
|                                              | (E+F) <sub>8</sub>                                                                               | 3.85       | 3.50       | B <sub>8</sub>            | *            |

— Not significant.

\* Significant at 5% level.

\*\* Significant at 1% level, or highly significant.

Some samples, including wheat, received "tender," "tough," and "normal" ratings on the same day. The wheat samples had a grand mean of 4.81 in comparison with a "normal" tenderness score of 5.0 and the soy samples ranged from 4.70 for the 4% level of one ethanol flour to 4.92 for the 4% level of the other. Because the deviation from the normal was so small, the test of significance was not applied to these mean scores.

*Soylike Flavor.* Judges readily distinguished between the wheat samples and any soy sample, as is shown by a comparison of the mean

scores in Table II (a and b). The mean score of 4.72 for wheat was significantly different from the mean score for the 4% and 8% levels of each of the soy samples ( $P = 0.01$ ). Differences were not significant between two flours of the same kind (Table IIa) except for the two hexane flours (A and B) at the 8% level. This difference suggests that the hexane process may vary from one processor to another.

The 8% level of each soy flour had significantly more soylike flavor than the 4% level (Table IIc) except for hexane A, the scores for which were 4.04 for the 4% and 3.94 for the 8% levels. None of the differ-

TABLE III  
STATISTICAL SIGNIFICANCE OF DIFFERENCES IN DESIRABILITY OF FLAVOR

| Comparisons                                  | Sample and % Level of Soy                                                                    | Mean Score | Mean Score | Sample and % Level of Soy | Significance |
|----------------------------------------------|----------------------------------------------------------------------------------------------|------------|------------|---------------------------|--------------|
| a) Within type at each level                 | A <sub>4</sub>                                                                               | 4.24       | 4.25       | B <sub>4</sub>            | —            |
|                                              | A <sub>8</sub>                                                                               | 4.12       | 3.91       | B <sub>8</sub>            | —            |
|                                              | C <sub>4</sub>                                                                               | 4.04       | 4.28       | D <sub>4</sub>            | —            |
|                                              | C <sub>8</sub>                                                                               | 3.59       | 3.98       | D <sub>8</sub>            | —            |
|                                              | E <sub>4</sub>                                                                               | 4.38       | 4.23       | F <sub>4</sub>            | —            |
|                                              | E <sub>8</sub>                                                                               | 4.27       | 4.04       | F <sub>8</sub>            | —            |
| b) Between wheat and soy flour at each level | Mean score of wheat (4.77) significantly different from mean score of each of soy samples.** |            |            |                           |              |
| c) Between levels within a type              | (A+B) <sub>4</sub>                                                                           | 4.24       | 4.01       | (A+B) <sub>8</sub>        | —            |
|                                              | (C+D) <sub>4</sub>                                                                           | 4.17       | 3.80       | (C+D) <sub>8</sub>        | **           |
|                                              | (E+F) <sub>4</sub>                                                                           | 4.30       | 4.15       | (E+F) <sub>8</sub>        | —            |
| d) Between types within and between levels   | (A+B) <sub>4</sub>                                                                           | 4.24       | 4.17       | (C+D) <sub>4</sub>        | —            |
|                                              | (A+B) <sub>8</sub>                                                                           | 4.24       | 4.30       | (E+F) <sub>4</sub>        | —            |
|                                              | (C+D) <sub>4</sub>                                                                           | 4.17       | 4.30       | (E+F) <sub>8</sub>        | —            |
|                                              | (A+B) <sub>8</sub>                                                                           | 4.24       | 4.15       | (E+F) <sub>8</sub>        | —            |
|                                              | (A+B) <sub>8</sub>                                                                           | 4.01       | 3.80       | (C+D) <sub>8</sub>        | —            |
|                                              | (A+B) <sub>8</sub>                                                                           | 4.01       | 4.15       | (E+F) <sub>8</sub>        | —            |
|                                              | (C+D) <sub>8</sub>                                                                           | 3.80       | 4.15       | (E+F) <sub>8</sub>        | *            |
|                                              | (E+F) <sub>8</sub>                                                                           | 4.30       | 4.01       | (A+B) <sub>8</sub>        | *            |

— Not significant.

\* Significant at 5% level.

\*\* Significant at 1% level, or highly significant.

ences between the scores for the 4% soy flour levels (Table IIId) were statistically significant, although there was a little less soylike flavor in the ethanol-extracted soy flour than in either of the other two types. At the 8% level, however, the full-fat flours differed significantly from one of the hexane flours (A) and the two ethanol flours (E and F), being more pronounced in soylike flavor than any of them. One of the hexane soy flours (B) had a significantly more soylike flavor than the ethanol flours E and F; whereas the other did not.

*Desirability of Flavor.* According to the judges, the bread baked

with all wheat flour had significantly more desirable flavor than any of the soy breads. The mean score for desirability of flavor of the wheat sample was 4.77 (Table IIIb) and the highest mean for any soy sample was 4.38 (E<sub>4</sub>, Table IIIa), indicating that in this study bread baked from wheat flour alone was preferred to that containing 4% or 8% of the three types of soy flours. There was no significant difference within 4% levels or within 8% levels of flours of the same type (Table IIIa). The 4% levels of the full-fat soy flours (C + D, Table IIIc) had a significantly more desirable flavor than the 8% levels;

TABLE IV  
STATISTICAL SIGNIFICANCE OF DIFFERENCES IN ACCEPTABILITY OF BREAD

| Comparisons                                  | Sample and % Level of Soy                                                                     | Mean Score | Mean Score | Sample and % Level of Soy | Significance |
|----------------------------------------------|-----------------------------------------------------------------------------------------------|------------|------------|---------------------------|--------------|
| a) Within type at each level                 | A <sub>4</sub>                                                                                | 4.25       | 4.30       | B <sub>4</sub>            | —            |
|                                              | A <sub>8</sub>                                                                                | 4.24       | 3.91       | B <sub>8</sub>            | —            |
|                                              | C <sub>4</sub>                                                                                | 4.12       | 4.36       | D <sub>4</sub>            | —            |
|                                              | C <sub>8</sub>                                                                                | 3.82       | 4.02       | D <sub>8</sub>            | —            |
|                                              | E <sub>4</sub>                                                                                | 4.42       | 4.27       | F <sub>4</sub>            | —            |
|                                              | E <sub>8</sub>                                                                                | 4.33       | 4.11       | F <sub>8</sub>            | —            |
| b) Between wheat and soy flour at each level | Mean scores of wheat (4.80) significantly different from mean score of each of soy samples.** |            |            |                           |              |
| c) Between levels within a type              | (A+B) <sub>4</sub>                                                                            | 4.28       | 4.06       | (A+B) <sub>8</sub>        | —            |
|                                              | (C+D) <sub>4</sub>                                                                            | 4.25       | 3.95       | (C+D) <sub>8</sub>        | **           |
|                                              | (E+F) <sub>4</sub>                                                                            | 4.34       | 4.21       | (E+F) <sub>8</sub>        | —            |
| d) Between types within and between levels   | (A+B) <sub>4</sub>                                                                            | 4.28       | 4.25       | (C+D) <sub>4</sub>        | —            |
|                                              | (A+B) <sub>8</sub>                                                                            | 4.28       | 4.34       | (E+F) <sub>4</sub>        | —            |
|                                              | (C+D) <sub>4</sub>                                                                            | 4.25       | 4.34       | (E+F) <sub>8</sub>        | —            |
|                                              | (A+B) <sub>8</sub>                                                                            | 4.28       | 4.21       | (E+F) <sub>8</sub>        | —            |
|                                              | (A+B) <sub>8</sub>                                                                            | 4.06       | 3.95       | (C+D) <sub>8</sub>        | —            |
|                                              | (A+B) <sub>8</sub>                                                                            | 4.06       | 4.21       | (E+F) <sub>8</sub>        | —            |
|                                              | (C+D) <sub>8</sub>                                                                            | 3.95       | 4.21       | (E+F) <sub>8</sub>        | *            |
|                                              | (E+F) <sub>4</sub>                                                                            | 4.34       | 4.06       | (A+B) <sub>8</sub>        | *            |

— Not significant.

\* Significant at 5% level.

\*\* Significant at 1% level, or highly significant.

whereas the 4% and 8% levels were not significantly different for the hexane and ethanol soy flours. When comparisons were made between types of soy flour within and between levels (Table IIId) it was found that the 4% and 8% levels of ethanol-extracted soy flour (E + F) were preferred to the 8% levels of hexane (A + B) and full-fat (C + D). There were no other significant differences.

*Acceptability of Bread.* The scores for acceptability of bread (Table IV) were slightly higher than those for desirability of flavor, indicating that factors other than flavor were considered in scoring for

acceptability. The acceptability scores, nevertheless, were very similar to those for desirability of flavor in that the wheat sample was significantly higher than any of the soy samples, the 4% level of full-fat was significantly higher than the 8% level, and the 4% and 8% levels of ethanol-extracted soy flour were preferred to the 8% levels of hexane and full-fat soy flours, respectively.

### Discussion

The baking studies reported herein indicate that bread of excellent loaf volume and crumb grain may be made from hard winter wheat flour and soy flour blends including up to 8% of the latter. To secure these desirable results, however, it is necessary to use larger quantities of potassium bromate in the baking formula than is customary. These results, together with the high water absorption requirements and buffering properties noted for the soy flours in blends with the hard winter wheat flour, are in agreement with those previously reported by Finney (3) for blends of hard spring wheat flour and soy flour.

Although excellent bread from the standpoint of loaf volume and crumb grain was made from the wheat-soy flour blends, these palatability studies indicated that the wheat bread had more desirable flavor than that baked from soy and wheat flour blends and that the bread made with 8% of full-fat soy flour was less desirable in flavor than that made with 4% of full-fat or with 8% of ethanol-extracted soy flour. These studies also indicated that full-fat soy flour is more easily detected than oil-free flours, particularly if as much as 8% is used in bread, and that 8% of soy flour, either oil-free or full-fat, is more easily detected in bread than 4%.

The flour used in these studies contained more protein than the average flour used by commercial bakers. Thus all the conclusions do not necessarily apply to results which might be obtained in commercial practice with flours containing appreciably less protein.

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# PRODUCTION OF MOLD AMYLASES IN SUBMERGED CULTURE

## II. FACTORS AFFECTING THE PRODUCTION OF ALPHA-AMYLASE AND MALTASE BY CERTAIN ASPERGILLI<sup>1, 2</sup>

HENRY M. TSUCHIYA, JULIAN CORMAN, and HAROLD J. KOEPEL

### ABSTRACT

The deleterious effect of low terminal pH in mold cultures on the yield of fungal alpha-amylase, previously reported by various investigators, appears to be due to the inactivation of the enzyme at low pH. Tests showed that fungal alpha-amylase becomes increasingly labile at pH values below 4.5. On the other hand, the stability of fungal maltase is fairly constant over a pH range of 4.2 to 7.25.

In the absence of calcium carbonate, the terminal pH of cultures of *Aspergillus niger* NRRL 337 can be controlled by adjustment of the concentrations of medium ingredients, namely, distillers' thin stillage solids derived from the alcohol fermentation of corn mash and corn meal. Increasing the thin stillage solids content of the medium results in the rise of terminal pH with a consequent increase in yield of alpha-amylase. Increasing the corn meal content of the medium results in a lowering of terminal pH. It also results in an increase in yield of maltase. By adjusting the concentrations of distillers' thin stillage solids and corn meal in the medium, it is possible to control to some degree, the yields of both alpha-amylase and maltase.

Incorporation of calcium carbonate in the medium in concentrations, previously recommended, results in a lowering in the yield of maltase. However, calcium chloride does not exhibit this deleterious effect. Inasmuch as the terminal pH can be controlled in the absence of calcium carbonate by using the proper amounts of thin stillage solids, it is recommended that calcium carbonate be eliminated from the medium used for production of fungal amylase.

Although variation in concentrations of distillers' thin stillage and corn meal in the medium affect the production of alpha-amylase and maltase in cultures of *Aspergillus niger* NRRL 330 and *A. oryzae* NRRL 458, the effect is not as marked as with carbohydrase production by *A. niger* NRRL 337.

The preparation of "fungi diastase" by the submerged culture propagation of *Aspergillus oryzae* in distillers' thin stillage and the utilization of such liquors as the converting agent in distillery operation was proposed by Woolner and Lassloff as early as 1909 (13). Until recently, however, little or no interest has been displayed in this country in the replacement of distillers' malt by fungal amylases

<sup>1</sup> Manuscript received January 2, 1950.

<sup>2</sup> Contribution from Northern Regional Research Laboratory, Peoria, Illinois. One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

produced in submerged culture. In order to obviate the shortcomings inherent in the Amylo process, Erb and Hildebrandt (3) modified the process by supplementing the mold amylase produced in submerged culture of *Rhizopus delemar* with a small amount of malt.

The complete replacement of malt with culture liquors of *Aspergillus niger* NRRL 337 produced by submerged culture processes was first reported by Van Lanen and LeMense (12). Since this communication, Adams *et al.* (1), Erb *et al.* (4) and the Northern Regional Laboratory investigators (5, 2, 11) have published their findings with *A. niger* NRRL 337. The culture liquor of this mold contains at least two carbohydrases, alpha-amylase and maltase. The former is responsible for the dextrinization of starch. Schwimmer (9) and Corman and Langlykke (2) demonstrated the significant role the latter plays in the production of fermentable sugars from starch. Although the possible presence of at least one other carbohydrase has been detected by Tsuchiya, Montgomery and Corman (11), this report is concerned with the factors affecting the yields of alpha-amylase and maltase in submerged culture.

### Material and Methods

*Production of Fungal Amylase Preparations.* Stock cultures of molds were maintained on a medium containing distillers' thin stillage derived from the alcohol fermentation of a corn mash (5% on solids basis or 1.5% on protein basis), soluble starch (2%), and agar (2%). After such stock cultures had sporulated, they were stored at 4°C.

The inoculum was prepared in the following manner: A 24-hr. slant culture was first prepared on the same medium as that used for stock cultures. A loopful of mold was then transferred to 100 ml. of the same medium, less agar, contained in 500 ml. Erlenmeyer flask. The flask was aerated on a reciprocal shaker for 24 hr.

Five ml. of the 24-hr. shake flask culture were used to inoculate 200 ml. of the fungal amylase production medium in 1 liter Erlenmeyer flasks containing variable amounts of corn meal and distillers' thin stillage as the sole ingredients. In some experiments calcium carbonate was also used. The exact medium composition will be given in detail later. The culture was aerated for 6 days at 30°C. on a reciprocal shaker. The mold mycelium was filtered off and the filtrate tested for alpha-amylase and maltase activity.

*Alpha-amylase Activity.* Alpha-amylase was determined by the Olson, Evans, and Dickson (7) modification of the Sandstedt, Kneen, and Blish (8) procedure. A unit of alpha-amylase is that amount of enzyme which dextrinizes 1 g. of beta-amylase treated starch in 1 hr. at 20°C.

**Maltase Activity.** A unit of maltase is that amount of enzyme which hydrolyzes 1 mg. of maltose monohydrate in 1 hr. at 30°C. The method depends on the observation that an increase of 78% in reducing power is obtained when maltose monohydrate is hydrolyzed to glucose under conditions given below. There is a stoichiometric relationship between the amount of enzyme and hydrolysis rate, within limits, when the rate is calculated from the difference in maltose hydrolyzed at 15 and 120 min.

**Reagents:**

(1) Acetate buffer (pH 4.4) solution, 6.0 *M*. 217 ml. of glacial acetic acid and 183 g. of anhydrous sodium acetate are diluted to one liter with water.

(2) Acetate buffer, 0.3 *M*, maltose substrate, 0.06 *M* solution. 2.35 g. of maltose monohydrate (92% pure as calculated on reducing value; e.g., Eastman Kodak Company product) and 5 ml. of acetate buffer (1), are diluted to 100 ml. with water.

(3) Sulfuric acid solution, 1*N*: sodium hydroxide solution, 1*N*; phenolphthalein indicator.

(4) Reagents for sugar estimation by method of Somogyi (10).

**Determination of Maltase Activity.** 5 ml. of fungal amylase preparation (culture filtrate) and 10 ml. of buffered substrate solution (2), both attempered to 30°C. (86°F.), are mixed in a test tube and held in a water bath at 30°C. After 15 min., a 3 ml. aliquot of the reaction mixture is transferred to a 100 ml. volumetric flask containing 3 ml. of 1 *N* sulfuric acid to stop the maltose hydrolysis by acid inactivation of the enzyme. After 120 min., a second 3 ml. aliquot of the reaction mixture is treated in similar manner.

After the acid inactivation of enzyme for 10 min., the acidified reaction mixtures are adjusted to the phenolphthalein end point with 1 *N* sodium hydroxide solution and made up to 100 ml. with water. 5 ml. aliquots are taken for analyses for reducing value (R.V.) by the method of Somogyi using the 20 min. heating period. By this procedure, the R.V. of the reaction mixture after 15 and 120 min. hydrolyses are obtained. The R.V. are measures of the glucose produced, the residual maltose, and the reducing sugars originally present in the enzyme preparation.

**Calculation:**

$a$  = R.V. of 15 min. reaction mixture.

$b$  = R.V. of 120 min. reaction mixture.

$$\frac{(b-a)}{0.78} \times (\text{glucose equivalent of Na}_2\text{S}_2\text{O}_3 \text{ solution} \times 1.78) \times 20 \times \frac{60}{105}$$

$$= \text{mg. maltose hydrolyzed per ml. of enzyme preparation per hr.}$$

*Precautions:*

(1) Hydrolysis rate values should be between 2 and 10 mg. maltose hydrolyzed per ml. enzyme preparation per hr. to be acceptable. Values in higher range are preferred.

(2) A pH of 4.4 must prevail in reaction mixture. Fungal amylase preparations highly buffered at pH values other than 4.4. must be adjusted to approximately this point before test.

(3) The copper reagent used in sugar analyses should be measured with a pipet rather than a buret.

**Results and Discussion**

*Effect of pH on Enzymes.* LeMense and his associates (5) showed that yields of alpha-amylase are lowered in cultures with low terminal pH. They recommended that calcium carbonate be incorporated as a

TABLE I

EFFECT OF pH ON STABILITY OF CARBOHYDRASES OF *Aspergillus niger* NRRL 337

| Culture Filtrate | Alpha-amylase | Maltase   |
|------------------|---------------|-----------|
| pH               | units/ml.     | units/ml. |
| 4.2              | 2.3           | 2.9       |
| 4.45             | 6.7           | 2.7       |
| 4.75             | 8.4           | 2.9       |
| 5.7              | 8.6           | 2.8       |
| 6.5              | 8.6           | 2.9       |
| 7.25             | 8.4           | 2.7       |

Temperature: 30°C.

Time: 18 hr.

Original alpha-amylase content: 8.6 units/ml.

Original maltase content: 2.8 units/ml.

buffering agent in the medium used for the propagation of molds for maximal yield of this enzyme. Inasmuch as the yield is a function of both stability and formation of the enzyme, the effect of pH on the stability of mold alpha-amylase and maltase of *A. niger* NRRL 337 was first studied. Culture filtrates were adjusted to various pH values between 4.2 and 7.25 with buffers and held at 30°C. for 18 hr. The data in Table I indicate that alpha-amylase was stable over a pH range of 4.75 to 7.25. However, some destruction of the enzyme occurred at pH 4.45 and considerable inactivation took place at 4.2. On the other hand, maltase was stable over the entire range tested. If alpha-amylase were stable over the entire range, such a fact would have suggested that the enzyme is not produced in culture of low pH. However, since it was inactivated at low pH values, the possibility existed that it may be produced and subsequently inactivated under acid conditions.

TABLE II

EFFECT OF INITIAL pH ON YIELDS OF CARBOHYDRASLS IN CULTURES OF *Aspergillus niger* NRRL 337

| pH   | Alpha-amylase | Maltase   |
|------|---------------|-----------|
|      | units/ml.     | units/ml. |
| 3.7  | 3.5           | 12.3      |
| 4.2  | 8.9           | 12.7      |
| 4.5  | 11.4          | 12.1      |
| 5.0  | 11.9          | 12.8      |
| 5.7  | 12.1          | 12.8      |
| 6.25 | 8.6           | 12.5      |

The effect of initial pH of the medium on enzyme yield was studied by varying the pH with 1 *N* sodium hydroxide or hydrochloric acid over the range of 3.7 to 6.25. *A. niger* NRRL 337 was grown in a 2% corn and 5% thin stillage solids medium to which calcium carbonate was not added. The results shown in Table II indicate that yields of alpha-amylase decreased when the initial pH was lowered below 4.5 or raised above 5.7. Since the enzyme is stable at pH values up to 7.25, the results together with those from the previous experiment indicate that alpha-amylase production is inhibited at the higher pH. On the other hand, maltase yields were constant over the entire range tested.

*Effect of Medium Composition on Terminal pH and Enzyme Yields.* Inasmuch as the results were at variance with those reported by LeMense and his associates (5) and also by Erb and his coworkers (4), namely, that calcium carbonate must be incorporated in the medium for adequate enzyme production, this phenomenon was examined more closely. It appeared that the pH had been controlled by the composition of our medium. Since it is frequently possible to control the pH in growing cultures by adjusting the amounts of carbon and

TABLE III

EFFECT OF MEDIUM COMPOSITION ON TERMINAL pH OF CULTURES OF *Aspergillus niger* NRRL 337

| Thin Stillage Solids | Corn %                           |     |     |     |
|----------------------|----------------------------------|-----|-----|-----|
|                      | 2                                | 5   | 7   | 10  |
|                      | Terminal pH of culture filtrates |     |     |     |
| %                    |                                  |     |     |     |
| 1                    | 3.5                              | 3.2 | 3.3 | 3.5 |
| 3                    | 4.7                              | 4.0 | 3.9 | 3.9 |
| 5                    | 4.5                              | 4.2 | 4.1 | 4.0 |
| 7                    | 4.6                              | 4.3 | 4.3 | 4.1 |

nitrogen sources (e.g., increasing the former lowers the pH whereas increasing the latter raises the pH), the effect of varying the corn and thin stillage solids contents of the medium was tested. The corn concentration was varied from 2 to 10% and the thin stillage solids level from 1 to 7%. The initial pH on the various media was adjusted to 5.2 with 1 *N* sodium hydroxide after sterilization. The effect on terminal pH of cultures of *A. niger* NRRL 337 is shown in Table III. As the concentration of corn is increased the terminal pH usually drops, but as the level of thin stillage solids is increased, the pH rises. The terminal pH in cultures containing calcium carbonate is approximately 5.0. This would appear to explain the discrepancy found between the data of LeMense and his associates (5) and ours. Pre-

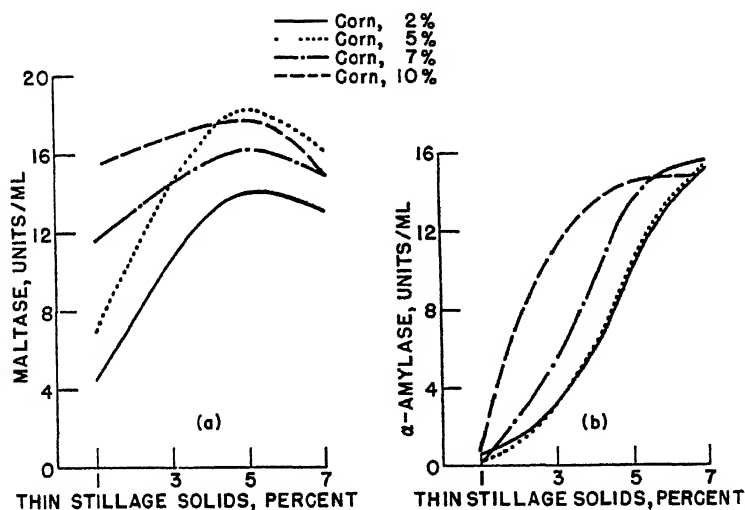


FIG 1. Effect of medium composition on maltase and alpha-amylase production of *Aspergillus niger* NRRL 337.

sumably, they worked with thin stillage in which the solids content was often low, and therefore, in the absence of calcium carbonate, the pH dropped to low values although low levels of corn were used. This resulted in a destruction of alpha-amylase. In the present experiments the thin stillage solids level of the mold culture medium was controlled by dilution of thin stillage sirup. Thus, by controlling the concentration of thin stillage solids contents, we controlled the terminal pH.

Perhaps more interesting than the effect of corn and thin stillage solids levels on the terminal pH of cultures was their effect on yields of maltase and alpha-amylase with *A. niger* NRRL 337. The yield of maltase at low levels of thin stillage solids is dependent upon the

concentration of corn as indicated in Fig. 1a. Although the use of higher concentrations of corn generally results in both the lowering of pH and the improvement in yields of maltase, evidence at hand indicates that factors other than pH are also involved. The beneficial effect of corn on the elaboration of fungal amylases has also been reported by Erb and his associates (4). As the stillage solids concentration is increased to optimal level, the effect of corn is minimized to some extent. As can be seen from Fig. 1b, thin stillage solids exert a marked effect on the yield of alpha-amylase. At 1% level, only trace amounts of this enzyme are elaborated, even in medium containing 10% corn. The low terminal pH values of these cultures probably destroyed this enzyme. As the thin stillage solids concentration is

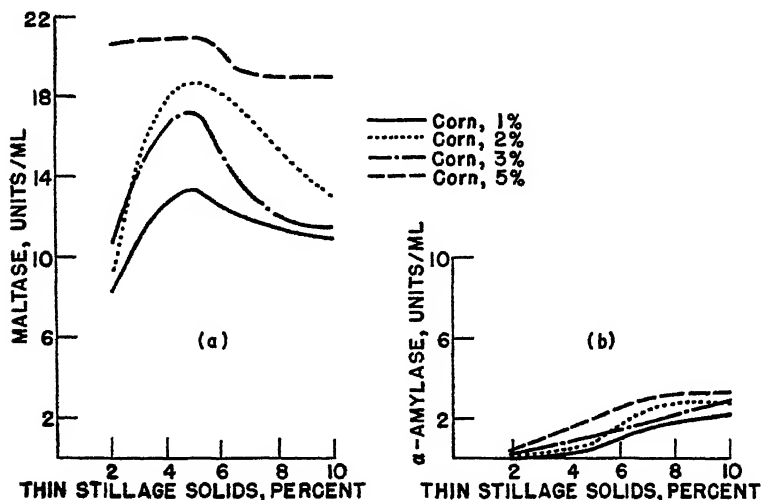


FIG. 2. Effect of medium composition of maltase and alpha-amylase production of *Aspergillus niger* NRRL 330.

increased, the terminal pH rises with a corresponding increase in the alpha-amylase content. That some other factor is also operative in the yield of alpha-amylase after the pH reaches 3.9, is seen by the fact that yields increase faster in media containing the higher concentrations of corn.

To see if enzyme yields could be controlled as readily with other organisms, this experiment was repeated with *A. niger* NRRL 330, a high maltase and low alpha-amylase producing mold, and *A. oryzae* NRRL 458, a low maltase and moderate alpha-amylase producing organism. Essentially the same effects are obtained with *A. niger* NRRL 330 (Figs. 2a and 2b). Increasing the level of corn to an optimal point raises the yield of maltase and increasing the concentra-

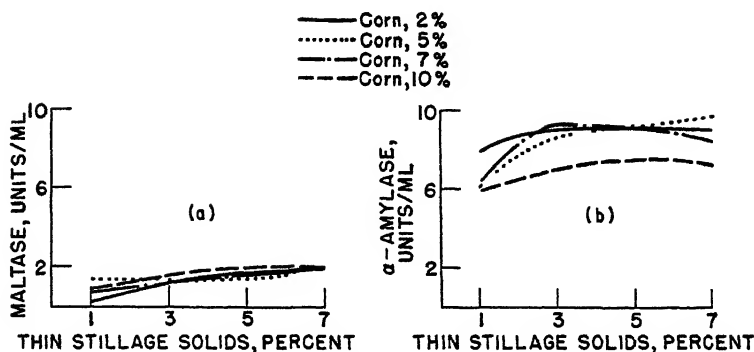


FIG. 3. Effect of medium composition on maltase and alpha-amylase production of *Aspergillus oryzae* NRRL 458.

tion of thin stillage solids raises the yield of alpha-amylase. On the other hand, varying the concentrations of corn and stillage solids has very little, if any, effect on the yields of these two enzymes by *A. oryzae* NRRL 458 (Figs. 3a and 3b).

**Effect of Calcium Carbonate on Enzyme Production.** The maltase yields found with *A. niger* NRRL 337 in 2% corn and 5% thin stillage solids medium appeared to be higher than those previously obtained with medium containing calcium carbonate. Therefore, controlled experiments were carried out to investigate the effect of calcium carbonate on enzyme production. The data from one of these experiments are shown in Figs. 4a and 4b. Calcium carbonate lowered the maltase yield at 0.05 *M* concentration and drastically inhibited the production of both alpha-amylase and maltase at 0.1 *M* level. It will be noted that at 0.05 *M* concentration, which is approximately equivalent to the 0.5% concentration recommended by LeMense and

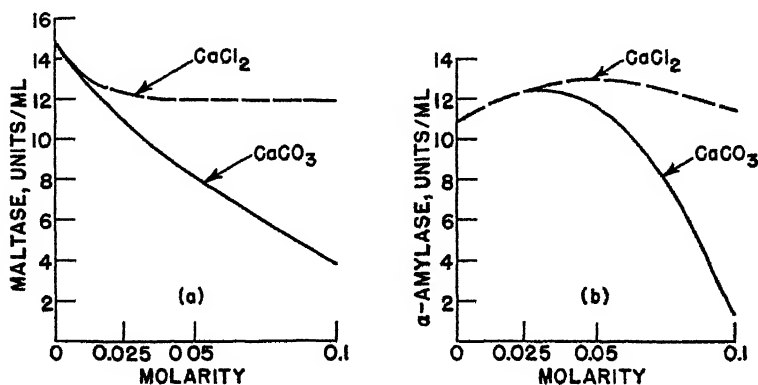


FIG. 4. Effect of calcium carbonate and calcium chloride on maltase and alpha-amylase production of *Aspergillus niger* NRRL 337.



his associates (who evaluated fungal amylase cultures on the basis of alpha-amylase potencies because the role of maltase in the production of fermentable sugars was not clearly understood at that time), the alpha-amylase yield is not lowered. Calcium chloride affects only slightly the yields of both maltase and alpha-amylase. Thus, it would appear that the calcium ion inhibits only slightly, if at all, the yield of maltase and benefits slightly the yield of alpha-amylase. The reason for the deleterious effect of calcium carbonate on the production of maltase is not yet known. It is not attributable to the calcium ion. Calcium carbonate is not required for the production of alpha-amylase, provided the thin stillage solids concentration is sufficient to buffer adequately cultures of *A. niger* NRRL 337. For maximal yields of maltase, calcium carbonate should be eliminated from the medium. Pilot-plant studies which will be reported elsewhere bear out the conclusions reached from these experiments.

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# THE EFFECT OF CRUST ON CHANGES IN CRUMBLINESS AND COMPRESSIBILITY OF BREAD CRUMB DURING STALING<sup>1,2</sup>

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## ABSTRACT

A series of experiments were made with normal and decrusted bread to determine if the moisture migration from the crumb to the crust had any effect upon the compressibility or crumbliness tests which are employed to follow the progress of crumb staling. Although moisture determinations revealed a loss of moisture from the crumb of the intact loaves amounting to 8% of the crumb moisture and a relatively constant moisture in the decrusted crumb during the course of the experiments, the rate and extent of changes in crumbliness and compressibility of the intact and decrusted bread were not appreciably different.

The changes in the crust of bread which occur during staling have generally been recognized as being caused by absorption of moisture. The sources of this moisture are known to be the internal crumb of the bread and, perhaps in some cases of high humidity, the atmosphere. In the case of modern-wrapped bread, the crumb is the more important source of moisture.

Geddes and his associates (1) have shown that appreciable quantities of water migrate from the crumb to the crust of bread which has been sealed in metal cans to prevent loss of moisture to the atmosphere. They reported the moisture content of the crust to increase from 15.4% for the fresh-baked loaf to 27.3% after six days of storage. Simultaneously, the moisture content of the central crumb decreased from 45.1% in the fresh bread to 37.0% in that stored for six days.

Moisture changes of this magnitude might be expected to decrease the compressibility of bread crumb and increase its crumbliness. Experiments were accordingly undertaken to determine whether this migration of moisture is a significant factor in results of such measurements on bread crumb after various intervals of storage.

## Materials and Methods

*Preparation and Storage of the Bread.* For each series of experiments, 18 loaves of bread were baked from dough of the following formula:

<sup>1</sup> Manuscript received December 15, 1949.

<sup>2</sup> Contribution from American Institute of Baking, Chicago, Illinois.

The study on which this article is based was made by the American Institute of Baking, under contract with the U. S. Department of Agriculture. The work was done under authority of the Research and Marketing Act.

|                        | %        |
|------------------------|----------|
| Flour                  | 100      |
| Sugar                  | 4        |
| Nonfat Dry Milk Solids | 3        |
| Shortening             | 3        |
| Salt                   | 2        |
| Yeast                  | 2        |
| Arkady                 | 0.25     |
| Calcium Propionate     | 1.0      |
| Water                  | Variable |

The results of preliminary studies with bread containing 0.3% of calcium propionate indicated a decrease in the moisture content of the crumb from the intact loaves of about 4% and an increase in crust moisture of about 9% in 96 hr. The crustless bread, however, was found to have a significantly higher moisture content than initially after the first 24 hr. due to growth of mold. Precautions were therefore necessary to prevent microbiological contamination of the samples used. Consequently, the calcium propionate in the bread formula was increased to 1% and a technique of preparing the decrusted bread was developed.

As soon as the bread cooled (about 1½ hrs. after removal from the oven), eight of the loaves were carefully stripped of the outer 1 cm. of crust and outer crumb by means of a thin-bladed knife, honed to razor sharpness, to minimize the possibility of mechanical damage to the fresh crumb. Handling of the crumb was avoided in an attempt to minimize its contamination with micro-organisms.

The crumb-sections and eight intact loaves were immediately placed in individual heat-sterilized, press-top tin containers and stored in a constant temperature cabinet at 35°C. The remaining two loaves were used immediately for moisture, compressibility, and crumbliness determinations.

*Moisture Determinations.* Moisture determinations were made daily upon the center crumb from the entire and the crustless loaves, and on the crust from the intact loaves. A section of center crumb weighing about 30 g. was weighed to the nearest centigram and air-dried overnight. The dehydration was then completed in a vacuum oven.

The moisture of the crust from the intact loaf was determined after the removal of as much crumb as possible. These determinations represented the moisture content of only the outer 3-4 mm. of crust which was composed essentially of the highly caramelized material.

*Compressibility Determinations.* Compressibility measurements were made upon three slices from each of two loaves of the bread. The crust was cut from the intact bread, and all slices were trimmed to provide sections 2.5 × 5 × 5 cm. in size.

The compressibility of these sections was determined by means of a penetrometer of the type specified by the American Society of Testing Materials for use with petroleum products, as reported by Sumner and Thompson (2). The penetration cone was replaced by a disk 3 cm. in diameter. The surface of the crumb-section was raised to this plate, which was then released to compress the crumb under a total load of 215 g. for 10 sec. The average of six such measurements was taken as the compressibility value.

*Crumbliness Determinations.* The crumbliness of the bread crumb was determined by a variation of a procedure reported by Bice and Geddes (1) and previously used by Sumner and Thompson (2).

Fifty grams of 12 mm. cubes were cut with the aid of a miter box and placed in a U. S. No. 4 sieve equipped with a catch pan and lid. A sheet metal disk 18 cm. in diameter and weighing 56 g. was placed on top of the cubes and shaken in a Precision Scientific Shaker for 15 min. The sifted crumb was weighed to the nearest centigram and taken as a measurement of crumbliness.

## Results

*Storage at 35°C.* The results of daily moisture, compressibility and crumbliness measurements on intact and crustless loaves stored at 35°C. for four days are presented in Table I. A significant migration of moisture from the crumb to the crust occurred, although its magnitude

TABLE I  
MOISTURE, COMPRESSIBILITY, AND CRUMBLINESS CHANGES IN BREADS  
Stored at 35°C.

| Age  | Bread with Crust |           |            |           |                      |                  | Bread without Crust |           |                      |                  |
|------|------------------|-----------|------------|-----------|----------------------|------------------|---------------------|-----------|----------------------|------------------|
|      | Moisture         |           |            |           | Compress-<br>ibility | Crumbli-<br>ness | Moisture            |           | Compress-<br>ibility | Crumbli-<br>ness |
|      | Crumb            |           | Crust      |           |                      |                  | Crumb               |           |                      |                  |
|      | Total<br>%       | Loss<br>% | Total<br>% | Gain<br>% |                      |                  | Total<br>%          | Loss<br>% |                      |                  |
| hrs. | Total<br>%       | Loss<br>% | Total<br>% | Gain<br>% | mm.                  | g.               | Total<br>%          | Loss<br>% | mm.                  | g.               |
| 1.5  | 44.9             | 0.0       | 25.1       | 0.0       | 12.1                 | 0.16             | 44.9                | 0.0       | 12.1                 | 0.16             |
| 24   | 43.6             | 2.9       | 30.5       | 21.5      | 8.7                  | 0.74             | 44.7                | 0.4       | 8.2                  | 0.85             |
| 48   | 43.0             | 4.2       | 31.7       | 26.3      | 5.6                  | 1.18             | 44.1                | 1.8       | 6.6                  | 1.02             |
| 72   | 42.4             | 5.6       | 32.7       | 30.2      | 4.5                  | 1.76             | 44.4                | 1.1       | 4.8                  | 1.62             |
| 96   | 41.4             | 7.8       | 32.9       | 31.0      | 4.2                  | 2.79             | 44.8                | 0.2       | 4.7                  | 4.69             |

was much less than that reported by Bice and Geddes (1). There was no significant change in the moisture content of the decrusted bread during the period of the experiment. In spite of the definite decrease in crumb moisture of the intact loaves, there was no difference between their compressibility and that of the crustless loaves when moisture

TABLE II  
MOISTURE, COMPRESSIBILITY, AND CRUMBLINESS CHANGES IN BREAD  
Stored at 24°C.

| Bread with Crust |            |           |            |           |                      |                  | Bread without Crust |           |                      |                  |  |
|------------------|------------|-----------|------------|-----------|----------------------|------------------|---------------------|-----------|----------------------|------------------|--|
| Age              | Moisture   |           |            |           | Compress-<br>ibility | Crumbli-<br>ness | Moisture            |           | Compress-<br>ibility | Crumbli-<br>ness |  |
|                  | Crumb      |           | Crust      |           |                      |                  | Crumb               |           |                      |                  |  |
| hrs.             | Total<br>% | Loss<br>% | Total<br>% | Gain<br>% | mm.                  | g.               | Total<br>%          | Loss<br>% | mm.                  | g.               |  |
| 1.5              | 42.9       | 0.0       | 20.0       | 0.0       | 9.1                  | 0.53             | 42.9                | 0.0       | 9.1                  | 0.53             |  |
| 24               | 40.5       | 5.6       | 25.8       | 29.0      | 4.0                  | 1.77             | 42.5                | 0.9       | 3.9                  | 2.05             |  |
| 48               | 39.5       | 7.9       | 27.5       | 37.5      | 1.9                  | 7.10             | 42.5                | 0.9       | 2.0                  | 8.42             |  |
| 72               | 39.5       | 7.9       | 30.0       | 50.0      | 1.9                  | 12.49            | 42.3                | 1.4       | 1.7                  | 9.62             |  |
| 96               | 39.4       | 8.2       | 30.7       | 53.5      | 1.5                  | 15.71            | 42.6                | 0.7       | 1.5                  | 13.16            |  |

migration was prevented. The crumbliness values were also remarkably similar except on the last day of measurement.

*Storage at 24°C.* The data obtained in a similar study on breads stored at 24°C. are presented in Table II. The moisture content of the crumb from the intact loaves was found to decrease at the same rate as occurred in that stored at 35°C., though there was a slight decrease in the rate of moisture increase in the crust. The compressibility and crumbliness values of the crustless and intact bread were not appreciably different at each time interval.

### Discussion

The results of these experiments indicate that the normal loss of moisture from bread crumb due to migration of water to the crust has little or no influence upon values obtained by applying the compressibility and crumbliness tests for the evaluation of staling. These two physical tests would appear to measure the effects of physical or chemical changes in the bread crumb which are known as staling and are not related to, or affected by, gross moisture migrations from one part of the loaf to another. In general, the migration of moisture from the crumb to the crust of bread caused an increase of as much as 50% of the initial moisture content of the latter in a 96-hr. period. During a like interval, a migration of approximately 8% of the center crumb moisture was found. In the case of bread from which the crust had been removed and which was stored to preclude evaporation, there was no change in crumb moisture.

Boutroux (3) was one of the first to theorize concerning the cause of bread staling. He postulated that during the staling of bread, moisture migration from crumb to crust caused the crumb to become dry and firm and the crust to become soft and leathery. He believed that

heating refreshed bread by driving the water from the crust back into the crumb, thus softening the crumb and producing a fresh, crisp crust. His theory has long since been discarded because of the inability of subsequent investigators to find a supersaturated solution of amylopectin in fresh bread, which was also a hypothesis included in his theory.

Data presented in this paper add further to the denial of the validity of Bouteux's theory. If either crumbliness or compressibility of crumb is a measure of bread staling, then staling is in no way dependent upon migration of water from the crumb to the crust, because the rate and extent of change of crumbliness and compressibility are as great in the crumb of bread in which migration of water is prevented as in the crumb of an intact loaf.

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## CHARACTERIZATION OF WHEAT GLUTEN II. AMINO ACID COMPOSITION<sup>1, 2</sup>

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### ABSTRACT

The amino-acid compositions of glutes prepared by ordinary washing procedures from 17 different flours were determined principally by microbiological assay methods. The compositions of the glutes were found to be essentially uniform, despite the wide range in type and source of the wheats and in the protein contents and baking characteristics of the flours from which the glutes were obtained. The average values, which are believed to be minimal because of the considerable amount of carbohydrate present during acid hydrolysis, are: ammonia, 4.5; alanine, 2.2; arginine, 4.7; aspartic acid, 3.7; cystine plus cysteine, 1.9; glutamic acid, 35.5; glycine, 3.5; histidine, 2.3; isoleucine, 4.6; leucine, 7.6; lysine, 1.8; methionine, 1.9; phenylalanine, 5.4; proline, 12.7; serine, 4.7; threonine, 2.6; tryptophan, 1.1; tyrosine, 3.1; valine, 4.7; as per cent of protein of theoretical nitrogen content of 17.5%.

<sup>1</sup> Manuscript received January 3, 1950. Paper I of this series was published in *Cereal Chem.* 24: 407-414 (1947).

<sup>2</sup> Contribution from Western Regional Research Laboratory, Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture, Albany, California.

Despite a number of attempts, no one has yet succeeded in finding a complete explanation for the differences in baking behavior of different flours. It is known that the *amount* of protein present is an important factor, yet flours with *equal* protein content often differ markedly in baking quality. As part of a systematic study of the factors responsible for these differences, we have now analyzed glutens from 17 flours of widely varying characteristics for their amino acid composition. No appreciable differences were found. Nevertheless, the analyses are presented, inasmuch as they are probably more nearly complete and accurate than those hitherto available.

### Materials and Methods

The flours used were straight-grade, unbleached flours experimentally milled from samples of pure varieties. The series included wheats of all major types and showed a wide range of protein content and baking behavior. Loaf volumes obtained in this Laboratory ranged from 435 cc. to 890 cc., using the baking formula of Finney and Barmore (5) with optimum bromate and mixing time.

Glutens were prepared from the flours by hand-washing doughs with the 0.1%, pH 6.8 phosphate buffer of Dill and Alsberg (4). The glutens contained, on the average, 88% of the flour nitrogen and 76% of the flour sulfur. Hydrolyzates for amino acid analysis were prepared by refluxing 2.5 g. of lyophilized gluten in 50 ml. of 6 *N* hydrochloric acid for 18 hr. Sufficient starch was added to each sample before hydrolysis so that all samples contained 12% nitrogen.<sup>3</sup> Most of the hydrochloric acid was removed by repeated concentration of the hydrolyzate under vacuum; after dilution to volume (100 ml.), the hydrolyzates were allowed to stand overnight before removal of the insoluble humin by filtration. Aliquots were neutralized for microbiological assay.

Tryptophan was determined in unhydrolyzed gluten by the method of Horn and Jones (6). Cystine-plus-cysteine values were obtained on hydrolyzates by a method (10) based on that of Vassel (14). Values for the remaining amino acids were obtained by microbiological assay of hydrolyzates with the following organisms: *Leuconostoc mesenteroides* P-60 (ATCC 8042) (arginine, aspartic acid, glycine, histidine, lysine, methionine, phenylalanine, proline, serine, and tyrosine); *Lactobacillus arabinosus* 17-5 (ATCC 8014) (glutamic acid, isoleucine, leucine, methionine, and valine); *Leuconostoc citrovorum* (ATCC 8081) (alanine and serine); *Streptococcus faecalis* R (ATCC 8043) (threonine).

<sup>3</sup> The different samples of washed glutens contained varying quantities of residual starch. The addition of starch to constant nitrogen content was designed to minimize variations in amino acid composition that might have resulted from destruction of amino acids during hydrolysis by reaction with carbohydrate break-down products.

BLE  
AMINO ACID CONTENTS OF WHEAT GLUTENS FROM FLOURS OF VARYING TYPES  
Amino Acid Values Expressed as Percentage of the Protein of Assumed 17.5% Nitrogen Content

| Wheat Variety      | Type   | Source   | Protein in flour <sup>1</sup><br>(N X 5.7) | Ammonia | Alanine | Arginine | Aspartic Acid | Cystine plus Cysteine | Glutamic Acid | Glycine | Histidine | Isoleucine |
|--------------------|--------|----------|--------------------------------------------|---------|---------|----------|---------------|-----------------------|---------------|---------|-----------|------------|
|                    |        |          | %                                          | %       | %       | %        | %             | %                     | %             | %       | %         | %          |
| Hymar              | W club | Wash.    | 5.7                                        | 4.2     | —       | 5.0      | 4.1           | 2.0                   | 34.9          | 3.2     | 2.3       | 4.6        |
| Orkwin             | WV     | Mich.    | 7.6                                        | 4.3     | —       | 4.9      | 3.9           | 2.0                   | 34.6          | 3.2     | 2.3       | 4.5        |
| Rex                | WV     | Wash.    | 7.8                                        | 4.3     | —       | 5.2      | 3.6           | 1.8                   | 35.2          | 3.5     | 2.4       | 4.4        |
| Purkof             | SPW    | Ind.     | 8.7                                        | 4.4     | —       | 4.8      | 3.6           | 1.9                   | 34.8          | 3.4     | 2.2       | 4.4        |
| Tennmarq           | HRW    | Kans.    | 8.8                                        | 4.5     | —       | 4.8      | 3.8           | 2.0                   | 34.3          | 3.9     | 2.3       | 4.5        |
| Hymar              | W club | Wash.    | 9.0                                        | 4.6     | —       | 4.5      | 3.6           | 1.9                   | 34.5          | 3.2     | 2.3       | 4.7        |
| Goetz              | SPW    | Ind.     | 9.1                                        | 4.4     | —       | 4.6      | 3.5           | 1.9                   | 35.5          | 3.4     | 2.2       | 4.4        |
| Boatz              | WS     | Wash.    | 9.5                                        | 4.5     | 2.1     | 4.5      | 3.6           | 1.8                   | 34.7          | 3.3     | 2.2       | 4.4        |
| Chickman           | HRW    | Kans.    | 9.6                                        | 4.4     | 2.3     | 4.8      | 3.9           | 1.9                   | 34.9          | 3.9     | 2.4       | 4.7        |
| Chickache          | HRW    | Kans.    | 9.7                                        | 4.6     | —       | 4.8      | 3.8           | 1.9                   | 34.9          | 3.8     | 2.3       | 4.5        |
| Turkey             | HRW    | Kans.    | 10.2                                       | 4.4     | —       | 4.7      | 3.9           | 2.0                   | 35.1          | 3.5     | 2.3       | 4.6        |
| Red Chief          | HRW    | Kans.    | 10.3                                       | 4.5     | —       | 4.8      | 3.9           | 2.1                   | 36.2          | 3.4     | 2.3       | 4.8        |
| Pentad             | Durum  | No. Dak. | 11.6                                       | 4.5     | 2.3     | 4.8      | 3.8           | 1.8                   | 35.1          | 3.2     | 2.4       | 4.8        |
| Premier            | HRS    | No. Dak. | 11.7                                       | 4.6     | —       | 4.6      | 3.7           | 1.8                   | 36.8          | 3.5     | 2.4       | 4.6        |
| Red Chief          | HRW    | Texas    | 12.9                                       | 4.5     | 2.2     | 4.6      | 3.5           | 2.0                   | 37.6          | 3.2     | 2.3       | 4.5        |
| Turkey             | HRW    | Texas    | 13.4                                       | 4.4     | 2.2     | 4.6      | 3.6           | 1.7                   | 36.9          | 3.4     | 2.3       | 4.4        |
| Thatcher           | HRS    | Mont.    | 14.2                                       | 4.6     | 2.1     | 4.3      | 3.5           | 1.8                   | 36.9          | 3.8     | 2.3       | 4.4        |
| Mean               |        |          |                                            | 4.5     | 2.2     | 4.7      | 3.7           | 1.9                   | 35.5          | 3.5     | 2.3       | 4.6        |
| Standard Deviation |        |          |                                            | ±.1     | ±.1     | ±.2      | ±.2           | ±.1                   | ±1.0          | ±.3     | ±.1       | ±.1        |



TABLE I—Continued

| Wheat Variety      | Type   | Source   | Leucine | Lysine | Methionine | Phenylalanine | Proline | Serine | Threonine | Tryptophan | Tyrosine | Valine |
|--------------------|--------|----------|---------|--------|------------|---------------|---------|--------|-----------|------------|----------|--------|
| Hymar              | W club | Wash.    | %       | %      | %          | %             | %       | %      | %         | %          | %        | %      |
| Yorkwin            | WW     | Mich.    | 8.0     | 2.1    | 2.1        | 5.3           | 12.6    | —      | 2.6       | 1.1        | 3.1      | 5.0    |
| Rex                | WW     | Wash.    | 8.0     | 2.0    | 2.0        | 5.4           | 13.3    | —      | 2.6       | 1.1        | 3.1      | 4.7    |
| Purkof             | SRW    | Ind.     | 7.6     | 2.1    | 1.9        | 5.1           | 12.9    | —      | 2.6       | 1.3        | 3.6      | 4.8    |
| Tennmarq           | HRW    | Kans.    | 7.2     | 1.9    | 1.7        | 5.0           | 12.4    | —      | 2.7       | 1.2        | 3.2      | 4.5    |
| Hymar              | W club | Wash.    | 7.2     | 1.9    | 1.8        | 5.1           | 11.9    | —      | 2.6       | 1.1        | 3.0      | 4.6    |
| Goens              | SRW    | Ind.     | 7.7     | 1.7    | 1.8        | 5.6           | 12.6    | —      | 2.6       | 1.1        | 3.1      | 4.7    |
| Baart              | WS     | Wash.    | 7.0     | 1.7    | 1.8        | 5.1           | 11.7    | —      | 2.5       | 1.0        | 2.9      | 4.4    |
| Chiefkan           | HRW    | Kans.    | 7.2     | 1.7    | 1.8        | 5.2           | 12.1    | 4.4    | 2.4       | 1.1        | 3.0      | 4.4    |
| Comanche           | HRW    | Kans.    | 7.8     | 1.8    | 1.9        | 5.3           | 12.6    | 4.8    | 2.7       | 1.1        | 3.2      | 4.7    |
| Turkey             | HRW    | Kans.    | 8.0     | 1.9    | 1.8        | 5.4           | 12.7    | —      | 2.8       | 1.1        | 3.1      | 4.8    |
| Red Chief          | HRW    | Kans.    | 8.0     | 1.9    | 2.0        | 5.4           | 13.3    | —      | 2.9       | 1.1        | 3.3      | 4.9    |
| Pentad             | Durum  | No. Dak. | 7.8     | 1.8    | 1.9        | 5.2           | 12.7    | —      | 2.6       | 1.1        | 3.0      | 4.7    |
| Premier            | HRW    | HRW      | 7.1     | 1.8    | 1.7        | 5.8           | 12.4    | 4.7    | 2.5       | 1.0        | 2.8      | 4.6    |
| Red Chief          | HRW    | Texas    | 8.0     | 1.7    | 2.0        | 5.7           | 13.3    | —      | 2.4       | 1.1        | 3.1      | 4.9    |
| Turkey             | HRW    | Mont.    | 7.4     | 1.8    | 1.8        | 5.7           | 13.0    | 5.0    | 2.4       | 1.1        | 3.1      | 4.7    |
| Thatcher           | HRW    | Mont.    | 7.5     | 1.7    | 1.9        | 5.7           | 13.2    | 5.0    | 2.5       | 1.1        | 3.3      | 4.7    |
| Mean               |        |          | 7.6     | 1.8    | 1.9        | 5.4           | 12.7    | 4.7    | 2.6       | 1.1        | 3.1      | 4.7    |
| Standard Deviation |        |          | ±.3     | ±.1    | ±.1        | ±.2           | ±.5     | ±.2    | ±.1       | ±.1        | ±.2      | ±.2    |

Details regarding the basal media, standards, and procedure will be described elsewhere (7). Amide nitrogen of the glutens was determined by a method previously described (11).

### Results and Discussion

The variety, type, and source of each of the 17 wheats and the amino acid contents of the corresponding glutens (calculated to 17.5% nitrogen<sup>4</sup> are presented in Table I.

The variation in amino acid contents among the glutens is quite small in most instances, although the range of values exceeds 20% of the mean for several of the amino acids. The coefficients of variability (standard deviation expressed as per cent of the mean), however, fall between 2.8 and 7.3%, which indicates that the dispersions of values around the means are quite small generally. Such variations are insignificant in face of the  $\pm 10\%$  limit of error often applied to microbiological amino acid assays.

Of particular interest are comparisons between pairs of flours of almost equal protein content but widely different baking characteristics. Two such pairs are: Turkey (Kansas) and Red Chief (Kansas); Comanche and Chiefkan. In each case, the latter of the pair is inferior as shown not only by experience in the baking industry but also confirmed by baking tests both in this Laboratory and elsewhere, with the particular flours. Loaf volumes obtained in this Laboratory for the Turkey (Kansas) and Red Chief (Kansas) flours were 665 cc. and 515 cc., respectively, and 630 cc. and 530 cc. for the Comanche and Chiefkan flours. Casual inspection reveals no trends upward or downward in the amount of any particular amino acid in the better member of the pairs. With Comanche and Chiefkan particularly, the analyses are almost identical.

The general uniformity of composition of the glutens is in agreement with the earlier reports of Blish (1) and Cross and Swain (3), who found no significant variation in amino acid composition among gliadins and glutenins prepared from different types of flour. However, McElroy *et al.* (9) analyzed whole wheats and obtained evidence that significant, although small, differences in lysine, arginine, and valine contents existed among different samples of a single variety (Marquis). These variations, of course, may reflect differences in the non-gluten nitrogenous components. Similarly, some of the variations in the series of analyses shown in Table I may reflect the presence of small amounts of non-gluten proteins. As an example, the slightly low amide nitrogen content of the glutens obtained from the three

<sup>4</sup> The 17.5% nitrogen corresponds to the factor, 5.7, traditionally used by cereal chemists to convert per cent nitrogen in wheat flour to per cent protein.

flours of lowest protein content can possibly be ascribed to the difficulties of washing these glutes uniformly free from the other proteins.

It should be emphasized that the amino acid contents found are minimum values, inasmuch as considerable destruction may have occurred as a result of the presence of the relatively large amounts of carbohydrate<sup>8</sup> substances during acid hydrolysis. Cystine, methionine,<sup>5</sup> tyrosine, tryptophan, and the basic amino acids are affected by such treatment (13, 8). Serine and threonine are known to be partially destroyed even in the absence of carbohydrate (12). Nevertheless,

TABLE II  
AVERAGE COMPOSITION OF WHEAT GLUTEN

| Constituent   | G. Amino Acid per 100 g. Protein <sup>1</sup> |                         | Moles Amino Acid per 10 <sup>3</sup> g. Protein <sup>1</sup> | Amino Acid Nitrogen of Total Nitrogen |
|---------------|-----------------------------------------------|-------------------------|--------------------------------------------------------------|---------------------------------------|
|               | Found                                         | Literature <sup>2</sup> |                                                              |                                       |
| Alanine       | 2.2                                           | 5.5                     | 25                                                           | 2.0                                   |
| Ammonia       | 4.5                                           | 4.5                     | 264                                                          | 21.2                                  |
| Arginine      | 4.7                                           | 4.3                     | 27                                                           | 8.6                                   |
| Aspartic acid | 3.7                                           | —                       | 28                                                           | 2.2                                   |
| Cystine       | 1.9                                           | 1.9                     | 8                                                            | 1.3                                   |
| Glutamic acid | 35.5                                          | 36.0                    | 241                                                          | 19.3                                  |
| Glycine       | 3.5                                           | —                       | 47                                                           | 3.7                                   |
| Histidine     | 2.3                                           | 2.4                     | 15                                                           | 3.6                                   |
| Isoleucine    | 4.6                                           | — <sup>3</sup>          | 35                                                           | 2.8                                   |
| Leucine       | 7.6                                           | — <sup>3</sup>          | 57                                                           | 4.6                                   |
| Lysine        | 1.8                                           | 2.1                     | 12                                                           | 2.0                                   |
| Methionine    | 1.9                                           | 3.3                     | 13                                                           | 1.0                                   |
| Phenylalanine | 5.4                                           | 2.0                     | 33                                                           | 2.6                                   |
| Proline       | 12.7                                          | 11.0                    | 110                                                          | 8.8                                   |
| Serine        | 4.7                                           | —                       | 45                                                           | 3.6                                   |
| Threonine     | 2.6                                           | 2.5                     | 21                                                           | 1.7                                   |
| Tryptophan    | 1.1                                           | 1.1                     | 5                                                            | 0.9                                   |
| Tyrosine      | 3.1                                           | 4.2                     | 17                                                           | 1.4                                   |
| Valine        | 4.7                                           | 3.0                     | 40                                                           | 3.2                                   |
| Total         | 108.5                                         |                         | 779 <sup>4</sup>                                             | 94.5                                  |

<sup>1</sup> Computed for a theoretical protein containing 17.5% nitrogen.

<sup>2</sup> Values cited by Blish, *Advances in Protein Chemistry*, II: 337-359 (1945).

<sup>3</sup> The literature value for the sum of leucine and isoleucine is 6.0.

<sup>4</sup> Number of moles of ammonia omitted from total.

approximately 95% of the total nitrogen is accounted for, as shown in Table II, without application of any correction to the analytical data. It can be inferred that such corrections, if known, would increase the total nitrogen accounted for to near 100%, and that no other amino acids were present, except in trace amounts.

No analyses were made for hydroxylysine or hydroxyproline. Rees (12) showed that hydroxylysine was absent from gliadin. Since

<sup>8</sup> Addition of starch to a gliadin preparation (nitrogen content, 17.0%) in amounts sufficient to reduce the nitrogen content to 16.0, 14.0, and 12.0% prior to hydrolysis caused apparent losses in cystine-plus-cystine of 4, 10, and 18%, respectively. Results with methionine were approximately the same. The total cystine-plus-methionine values reported in the tables account for an average of 81% of the total gluten sulfur.

there are no satisfactory methods for determining hydroxyproline, its possible presence was not investigated. However, the occurrence of these amino acids in proteins is believed to be limited to those related to collagen.

The mean values for many of the amino acids agree well with those compiled by Blish (2) (Table II). Our values for phenylalanine, leucine-plus-isoleucine, and valine are considerably higher and for alanine,<sup>6</sup> methionine, and tyrosine significantly lower than those previously available.

#### Acknowledgments

We are indebted to Mr. Roy K. Durham and the Millers National Federation for 13 of the flour samples used in this study. These samples were milled by Pillsbury Mills, Inc., Minneapolis. Baking data were supplied by Mr. G. Moen, General Mills, Inc., Minneapolis. Three samples of flour with baking data were furnished by the Bureau of Plant Industry, Soils, and Agricultural Engineering, Manhattan, Kansas. The remaining sample of flour was obtained through the courtesy of the Oregon Wheat Commission.

The authors wish also to acknowledge the careful technical assistance of Mrs. F. C. Marsh and Mrs. P. A. Thompson with the microbiological assays.

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<sup>6</sup> A comparison of the results of the microbiological method used here with other methods on reference proteins (7) suggests that the alanine values may be only two-thirds, approximately, of the true values.

## COMMUNICATIONS TO THE EDITOR

### The Niacin and Pantothenic Acid Content of Normal and Moldy Corn

DEAR SIR:

In our investigations of factors which affect the niacin and pantothenic acid content of corn hybrids it was found that immature corn (unpublished) had a higher content of these two vitamins than the same varieties when normally mature. Corn of high moisture content, irrespective of whether it is mature or immature, when stored may become moldy. The appearance of mold in both cases is a frequent occurrence under ordinary storage conditions.

In order to obtain more detailed information on the variation in the niacin and pantothenic acid content of normal and moldy corn, ten samples of apparently normal corn and thirteen samples of visibly moldy corn of the same varieties and grown in different locations were assayed for these vitamins by the microbiological method previously described (1). There was no way of knowing at that time whether the moldy corn was more immature than the apparently normal corn or vice versa. However, more recent results indicate that both groups of samples were mature but the moldy sample had a higher moisture content at some time or they would not have become moldy. The niacin content of the normal (non-moldy) corn varied from 19.7 to 31.2 (mean 24.1)  $\mu\text{g/g}$ , while the moldy samples varied from 21.2 to 37.7 (mean 30.00)  $\mu\text{g/g}$ . The results for pantothenic acid varied from 3.4 to 6.3 (mean 4.8)  $\mu\text{g/g}$  for normal (non-moldy) corn, while the moldy corn varied from 4.1 to 7.8 (mean 5.9)  $\mu\text{g/g}$ . These results are based on an air dried moisture content of about 10%. Where the samples were covered with a heavy mold the variation between normal and moldy corn, as described, was as much as 15  $\mu\text{g/g}$  of niacin and 3.0  $\mu\text{g/g}$  of pantothenic acid.

In order to obtain further information, samples of known mature corn were divided into two parts. One part was assayed as found. The moisture content of the other part was sufficiently increased and then incubated in covered enamel pans at room temperature (23°–25° C.) for about three days. The seeding of the samples was from the normal spores of the air. The moldy samples were then air dried to about 10% of moisture. The results for niacin and pantothenic acid on an air-dry basis were as follows:

| No. | Sample       | Niacin<br>μg/g | Pantothenic acid<br>μg/g |
|-----|--------------|----------------|--------------------------|
| 1   | Normal corn  | 25.8           | 5.7                      |
| 1A  | Same moldy   | 50.3           | 9.5                      |
| 2   | Normal moldy | 27.0           | 5.6                      |
| 2A  | Same moldy   | 52.8           | 7.8                      |

These results show that when mold was artificially induced on normal corn it contained significantly higher niacin and pantothenic acid content than normal corn samples, indicating that the mold was at least part of the factor or factors involved in the above results.

Several investigators (2, 3) have shown that various fungi or microorganisms synthesize niacin and pantothenic acid and the above results appear to confirm these. However, we wished to obtain additional information on the synthesis of niacin and pantothenic acid by growing mold on a vitamin free medium. *Rhizopus* sp.\* was isolated aseptically from the inside of the grain of moldy corn and grown in cotton-plugged erlenmeyer flasks containing the sterile vitamin-free medium. The medium contained 10 g. potassium nitrate, 5 g. potassium dihydrogen phosphate, 2.5 g. magnesium sulfate, 50 g. sucrose, and 2.0 g. vitamin-free casein hydrolysate per l. The incubation was for a period of about 3-4 days, when a sufficient amount of mycelia were obtained. These were collected, dried, and assayed for niacin and pantothenic acid with the following results, expressed on an air-dry basis:

| Sample no. | Niacin<br>μg/g | Pantothenic acid<br>μg/g |
|------------|----------------|--------------------------|
| 1          | 159.0          | 74.8                     |
| 2          | 154.7          | 74.7                     |

These data show that this particular mold has the ability to synthesize niacin and pantothenic acid to a high degree. These findings offer an explanation for the increased niacin and pantothenic acid content of moldy corn over and above that of normal (non-moldy) corn.

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February 27, 1950

\* Several species of *Rhizopus* were isolated from the moldy corn. The particular species used in this work was found to conform to the description of *R. nigricans* as given by Gilman (4) with respect to habit of growth and size of sporangia and spores.

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## Preparation of "Gluten" from Barley and Rye

SIR:

It is well known that, when a wheat-flour dough is manipulated under a stream of water, the starch is washed away and a cohesive, visco-elastic mass called gluten is obtained. This product contains about 80% of protein ( $N \times 5.7$ ), together with some carbohydrates, lipids, etc. Lusena (*Cereal Chem.* 27: 167-178 (1950)) has recently shown that a similar product can be prepared from wheat flour by dispersing the protein complex in 0.005 *N* acetic acid, removing the starch, etc., by centrifuging, and reprecipitating the gluten by careful neutralization.

Heretofore, no products similar to wheat gluten have been prepared from any other cereal grain by ordinary washing; nor have we been able to prepare them by the Lusena method. But, after prior water extraction, and by using 0.01 *N* formic acid rather than acetic acid, "glutens" have now been prepared from both barley and rye flour. By comparison with wheat gluten, these new products are tougher, and less elastic, cohesive, and sticky. Mechanical working tends to disintegrate them. They are reminiscent of glutens prepared from low-protein flour of inferior quality. By the same procedure, oat flour has as yet yielded only a clay-like product with no elastic properties.

A comparative study of the chemical and physical properties of these "glutens," and of the relations of these properties to extraction methods, is in progress. It may well yield results of value in elucidating the basic structure of the protein components of bread dough.

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March 18, 1950

## BOOK REVIEWS

**Advances in Agronomy.** Vol. I. Edited by A. G. Norman. 439 pp. Academic Press Inc. New York, New York. 1949. Price \$7.50.

The volume contains 10 chapters, each dealing with some phase of agronomic research or practice and each prepared by a recognized authority or authorities. Soil-crop relationships are emphasized throughout. Of the 10 chapters, six deal with soil in relation to plant growth and four with crop production. Each chapter includes a list of references which brings the subject matter up-to-date. These lists will provide a convenient source of reference material.

Hayward and Wadleigh of the U. S. Regional Salinity and Rebedoux Laboratory, Riverside, California, deal with saline and alkali soil-plant relationship in a thorough and comprehensive manner. Saline and alkali soils are differentiated and the physiological basis of salt and alkali tolerance of plants discussed. Specificity in salt tolerance for various species and varieties of crop plants are considered in some detail.

The second chapter on new fertilizers and fertilizer practices was written by Jones and Rogers of the T.V.A., Knoxville, Tennessee, who are in an excellent position to deal with the subject. Major attention is given to the development of new fertilizer materials and improved fertilizer practices. The chapter will be of interest not only to agronomists but to the industry as well.

Martin G. Weiss of Iowa State College, Ames, authors a chapter on soybeans. The treatment of the subject is comprehensive in that it deals with all phases of the crop, including production, utilization, culture, and improvement by breeding and testing. The crop has several important effects upon the soil and, at the same time, the soil is an important factor in the production of the crop. Disease and insect controls are discussed.

A fourth chapter on the clay minerals of soil by J. E. Gieseking, University of Illinois, Urbana, deals both with mineralogical and plant relationships of this rather intricate subject. The article is exceptionally well done and will be of interest to soil scientists and those agronomists interested in fertility and plant nutrition problems.

Wm. J. White prepared the chapter on alfalfa improvement. This is a very informative article and presents the matter of seed setting and production in a clear and interesting fashion. His discussion of the not too well understood question of pollination and pollinating insects is worth careful reading. The author emphasizes the importance of breeding disease resistant varieties.

The chapter on Soil Micro-organisms and Plant Roots by Francis E. Clark, U.S.D.A. and Iowa Agricultural Experimental Station develops the interesting relationship existing between plants roots and soil microorganisms. The approach to the subject is one not frequently seen and for this reason is deserving of a careful reading.

A. S. Crafts and W. A. Harvey, University of California, deal with weed control. They discuss conventional methods as well as the newer methods of chemical control. In the latter they deal with a wide variety of materials and their herbicidal action. Their thorough treatment of the subject will be of interest both to agronomists and the manufacturers of weed killers.

The chapter on Boron in Soils and Crops by K. C. Berger, University of Wisconsin, describes methods of determination of the element in both soils and crops, the availability of soil boron and the boron requirements of crop plants. It includes an up-to-date review of the literature on the subject.

Ora Smith, Cornell University, has prepared a most excellent chapter on potato production. It includes a discussion of the improvement of the crop by breeding, the control of weeds, the requirements of the crop for nutrients, and methods of soil management most adaptable to the production of the crop. Included also are discussions of disease and insect control. This chapter should be of much interest to potato growers.

The subject of phosphorus fixation in soils is discussed by L. A. Dean, U. S. Department of Agriculture. Many factors are known to be involved in phosphorus fixation and an extraordinarily large amount of research has been devoted to the problem. The author has done an excellent job of bringing these researches together and summarizing them.



The volume as a whole is extremely well done and should be of wide interest to soil scientists, agronomists, manufacturers, and growers. The general excellence of Vol. I presages well for Vol. II which is to appear late in the year.

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**Chemical Engineers' Handbook.** Edited by John H. Perry. XV and 1942 pp  
McGraw Hill Book Company Inc., New York. Third ed. 1950. Price \$15.00.

In preparing the third edition of this standard reference work, the first and second editions of which were published in 1934 and 1941 respectively, the editor was assisted by an advisory board of 15 chemical engineers and 141 contributors comprising specialists in various phases of chemical engineering.

In the preface to the current edition, the editor points out that the normal peacetime rate of progress in the theory and practice of chemical engineering has been so great during the prewar, war, and postwar years that it was necessary to rewrite most of the major sections of the handbook.

The book contains sections covering the following topics: mathematical tables and weights and measures; mathematics; physical and chemical data; physical and chemical principles; flow of fluids; heat transmission; evaporation; general theory of diffusional operations; distillation and sublimation; gas absorption; solvent extraction and dialysis; humidification, dehumidification, and cooling towers and spray ponds; drying; adsorption; mechanical separations; size reduction and size enlargement; mixing of material; high-pressure technique; process control; movement and storage of materials; materials of construction; fuels; furnace and kilns; power generation and mechanical power transmission; refrigeration; plant location; electricity and electrical engineering; electrochemistry; accounting and cost finding; and safety and fire protection.

The new material which has been added in the present edition includes general theory of diffusional operations; furnaces and kilns; size enlargement; azeotropic distillation; multi-component distillation; extractive distillation; molecular distillation; and dialysis while the following chapters have been deleted: reports and report writing; indicators, qualitative analysis; and organic chemistry. This edition is printed in a larger page size (approximately 7 by 10 inches) which not only permitted the thickness of the book to be reduced but also allowed the use of larger sizes of graphs and illustrations which has increased the clarity. The book is printed on good paper, is well indexed and is remarkably free from typographical errors.

The edition provides a dependable source of information on principles, data, and practice in the various branches of chemical engineering and will be of inestimable value to cereal chemists, particularly those concerned with process development and control.

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## Cereal Chemistry

### EDITORIAL POLICY

*Cereal Chemistry* publishes scientific papers dealing with raw materials, processes, or products of the cereal industries, or with analytical procedures, technological tests, or fundamental research, related thereto. Papers must be based on original investigations, not previously described elsewhere, which make a definite contribution to existing knowledge.

*Cereal Chemistry* gives preference to suitable papers presented at the Annual Meeting of the American Association of Cereal Chemists, or submitted directly by members of the Association. When space permits, papers are accepted from other scientists throughout the world.

The papers must be written in English and must be clear, concise, and styled for *Cereal Chemistry*.

Manuscripts for publication should be sent to the Editor in Chief. Advertising rates may be secured from and subscriptions placed with the Managing Editor, University Farm, St. Paul 1, Minnesota. Subscription rates, \$9 00 per year Foreign postage, 50 cents extra. Single copies, \$2 00; foreign, \$2.10.

### SUGGESTIONS TO AUTHORS

**General.** From January 1, 1948, an abstract will be printed at the beginning of each paper instead of a summary at the end, references will be numbered to provide the option of citing by number only, and date of receipt, author's connections, etc., will be shown in footnotes. Except on these points, authors will find the last volume of *Cereal Chemistry* a useful guide to acceptable arrangements and styling of papers. "On Writing Scientific Papers for Cereal Chemistry" (*Trans. Am. Assoc. Cereal Chem.* 6: 1-22, 1948) amplifies the following notes.

Authors should submit two copies of the manuscript, typed double spaced with wide margins on 8½ by 11 inch white paper, and all original drawings or photographs for figures. If possible, one set of photographs of figures should also be submitted. Originals can then be held to prevent damage, and the photographs can be sent to reviewers.

**Titles and Footnotes.** Titles should be specific, but should be kept short by deleting unnecessary words. The title footnote shows "Manuscript received . . ." and the name and address of the author's institution. Author footnotes, showing position and connections, are desirable although not obligatory.

**Abstract.** A concise abstract of about 200 words follows title and authors. It should state the principal results and conclusions, and should contain, largely by inference, adequate information on the scope and design of the investigation.

**Literature.** In general, only recent papers need be listed, and these can often be cited more advantageously throughout the text than in the introduction. Long introductory reviews should be avoided, especially when a recent review in another paper or in a monograph can be cited instead.

References are arranged and numbered in alphabetical order of authors' names and show author, title, journal, volume, first and last pages, and year. The list is given at the end of the paper. Reference numbers must invariably be cited in the text, but authors' names and year may be cited also. Abbreviations for the names of journals follow the list given in *Chemical Abstracts* 40: I-CCIX. 1946.

**Organization.** The standard organization involves main sections for abstract, introduction, materials, methods, results, discussion, acknowledgments, and literature cited. Alternately, a group of related studies, each made with different materials or methods, may require a separate section for each study, with subsections for materials and methods, and for results, under each section. Center headings are used for main sections and italicized run-in headings for subsections, and headings should be restricted to these two types only.

**Tables.** Data should be arranged to facilitate the comparisons readers must make. Tables should be kept small by breaking up large ones if this is feasible. Only about eight columns of tabular matter can be printed across the page. Authors should omit all unessential data such as laboratory numbers, columns of data that show no significant variation, and any data not discussed in the text. A text reference can frequently be substituted for columns containing only a few data. The number of significant figures should be minimized. Box and side heading should be kept short by abbreviating freely; unorthodox abbreviations may be explained in footnotes, but unnecessary footnotes should be avoided. Leader tables without a number, main heading, or ruled lines are often useful for small groups of data.

Tables should be typed on separate pages at the end of the manuscript, and their position should be indicated to the printer by typing "(TABLE I)" in the appropriate place between lines of the text (Figures are treated in the same way.)

**Figures.** If possible, all line drawings should be made by a competent draftsman. Traditional layouts should be followed: the horizontal axis should be used for the independent variable; curves should be drawn heaviest, axes or frame intermediate, and the grid lines lightest; and experimental points should be shown. Labels are preferable to legends. Authors should avoid identification in cut-lines to be printed below the figure, especially if symbols are used that cannot readily be set in type.

All drawings should be made about two to three times eventual reduced size with India ink on white paper, tracing linen, or blue-lined graph paper; with any other color, the unsightly mass of small grid lines is reproduced in the cut. Lettering should be done with a guide using India ink; and letters should be  $\frac{1}{16}$  to  $\frac{1}{8}$  inch high after reduction.

For difficult photographs, a professional should be hired or aid obtained from a good amateur. The subject should be lighted to show details. A bright print with considerable contrast reproduces best, and all prints should be made on glossy paper.

All original figures should be submitted with one set of photographic reproductions for reviewers, and each item should be identified by lightly writing number, author, and title on the back. Cut-lines (legends) should be typed on a separate sheet at the end of the manuscript. "Preparation of Illustrations and Tables" (*Trans. Am. Assoc. Cereal Chem.* 3: 69-104, 1945) amplifies these notes.

**Text.** Clarity and conciseness are the prime essentials of a good scientific style. Proper grouping of related information and thoughts within paragraphs, selection of logical sequences for paragraphs and for sentences within paragraphs, and a skillful use of headings and topic sentences are the greatest aids to clarity. Clear phrasing is simplified by writing short sentences, using direct statements and active verbs, and preferring the concrete to the abstract, the specific to the general, and the definite to the vague. Trite circumlocutions and useless modifiers are the main causes of verbosity; they should be removed by repeated editing of drafts.

**Editorial Style.** A.A.C.C. publications are edited in accordance with *A Manual of Style*, University of Chicago Press, and *Webster's Dictionary*. A few points which authors often treat wrongly are listed below:

Use names, not formulas, for text references to chemical compounds. Use plural verbs with quantities (6.9 g. were). Figures are used before unit abbreviations (3 ml.), and % rather than "per cent" is used following figures. All units are abbreviated and followed by periods, except units of time, which are spelled out. Repeat the degree sign ( $5^{\circ}$ - $10^{\circ}$  C.). Place 0 before the decimal point for correlation coefficients ( $r = 0.95$ ). Use \* to mark statistics that exceed the 5% level and \*\* for those that exceed the 1% level; footnotes explaining this convention are no longer required. Type fractions on one line if possible, e.g.,  $A/(B + C)$ . Use lower case for farinograph, mixogram, etc., unless used with a proper name, i.e., Brabender Farinograph. When in doubt about a point that occurs frequently, consult the Style Manual or the Dictionary.

# CEREAL CHEMISTRY

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## STUDIES OF THE EFFECTS OF POLYOXYETHYLENE MONOSTEARATE ON FLOUR AMYLASE ACTIVITY<sup>1,2</sup>

L. F. MARNETT and R. W. SELMAN

### ABSTRACT

Polyoxyethylene monostearate increases amylograph maximum paste viscosity of wheat flour pastes to a varying degree, dependent upon the amount of ester added and the alpha-amylase content of the flour. A significant increase in maximum viscosity is produced by polyoxyethylene monostearate in the absence of alpha-amylase. The effect is likewise manifested when the agent is added to pastes made from corn starch and waxy maize.

In amounts less than 0.25% polyoxyethylene monostearate only slightly retarded the dextrinization time with alpha-amylodextrin substrate, but higher percentages prevented complete hydrolysis even after 30 hrs. of incubation.

Maltose production from soluble starch solutions was decreased only when very high levels of amylase were employed in conjunction with abnormally high percentages of polyoxyethylene monostearate.

Favor and Johnson (5), (6), have shown that the inclusion of polyoxyethylene monostearate (hereinafter referred to as POEMS), in the bread formula decreased the rate at which bread crumb became firm in storage. These workers have reported that POEMS has little effect on gas production in fermenting doughs and on proofing.

The experiments reported herein were conducted to determine whether POEMS has any influence on amylase activity under conditions comparable with those which exist in bread making.

The composition of polyoxyethylene monostearate has been described by the aforementioned authors (5). The product used throughout the following investigations had an average molecular weight corresponding to the nonaethylene glycol monostearate.

### Procedures and Results

Anker and Geddes (2), Brown and Harrel (3), and Selman and Sumner (10) have described the utility of the amylograph in studying

<sup>1</sup> Manuscript received December 23, 1949. Presented at the annual meeting, May, 1949.

<sup>2</sup> Contribution from the C. J. Patterson Company, Kansas City, Missouri.

the pasting characteristics of flour and of various starches. Favor and Johnson (5) employed the amylograph to show the stabilizing effect of POEMS on starch gels.

*Effect on Wheat Flour Pastes.* The amylograph was used to measure the effect of POEMS on the paste viscosities of malt-supplemented

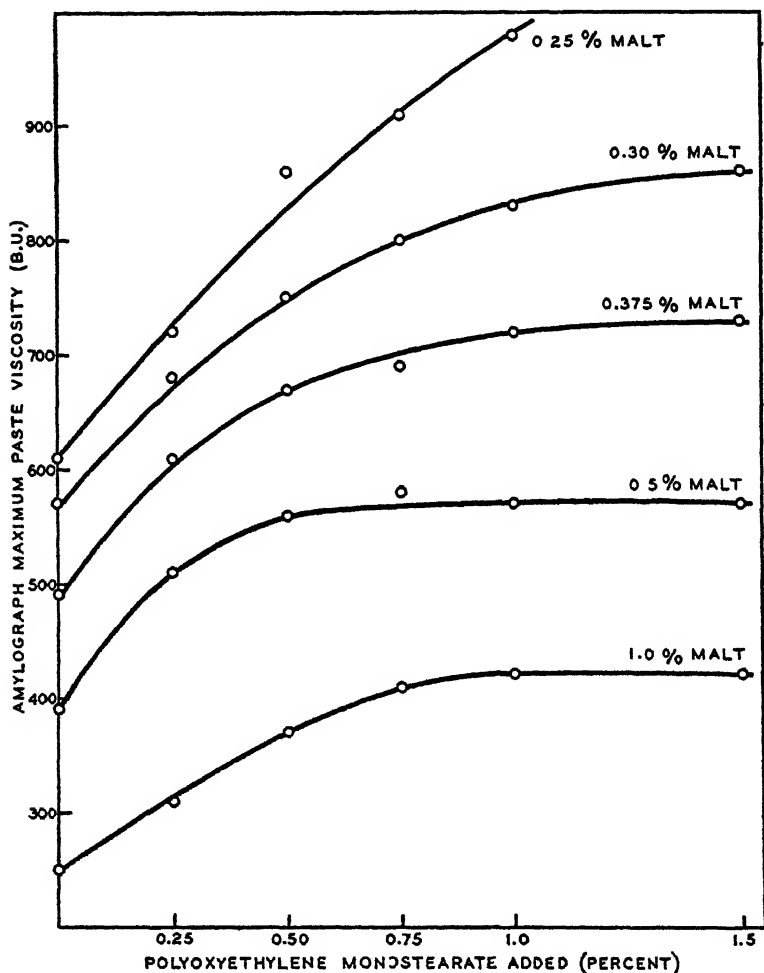


Fig. 1. The inter-relationship of polyoxyethylene monostearate and barley malt with amylograph maximum paste viscosity.

flour since the temperature increase in the amylograph is closely related to that which occurs in dough during the initial stages of baking, when starch conditioning is a major factor. Maximum paste viscosity is regarded as a measure of the starch liquifying effect of alpha-amylase.

Increments of barley malt were added to a commercially milled, non-diatated flour in order to obtain flours of various amylase levels. Varying amounts of POEMS were added to the flour-water suspensions by pipetting appropriate volumes of a 2.5% aqueous POEMS suspension and agitating to obtain adequate dispersion. The amylograph used in these studies had a temperature increase of 1.5°C. per min. The method used was that of Selman and Sumner (10), in which the amylograph is employed to determine starch paste viscosity as a function of thermal gelatinization and amylolysis.

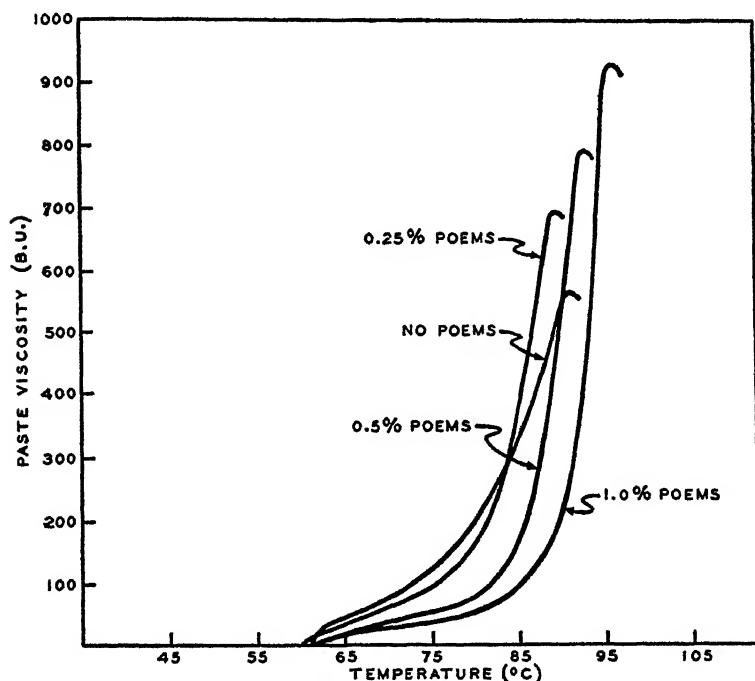


Fig. 2. Effect of polyoxyethylene monostearate on amylograph paste viscosity of a non-diatated flour.

The results shown in Fig. 1 demonstrate that POEMS increased maximum paste viscosity markedly when added in amounts as low as 0.25%. Equivalent increases in maximum viscosity were produced when 0.5% and 0.75% of the ester were present, but the increases with larger additions diminished in magnitude with each additional increment of POEMS.

The increases in maximum paste viscosity produced by POEMS indicate either alpha-amylase inhibition or modification of the starch substrate.

*Effect on Non-Diastated Flours.* To determine the reason for the viscosity increases observed in the foregoing instances, measurements were made of the pasting properties of unmalted flour. The amylograph procedure employed was again that of Selman and Sumner (10), except that 65 g. of flour were substituted for the customary 100 g., in order that the entire viscosity curve would fall within the recording range of the instrument.

This procedure was repeated in the presence of 20 p.p.m. of mercuric chloride to insure complete inactivation of traces of the amylases (4).

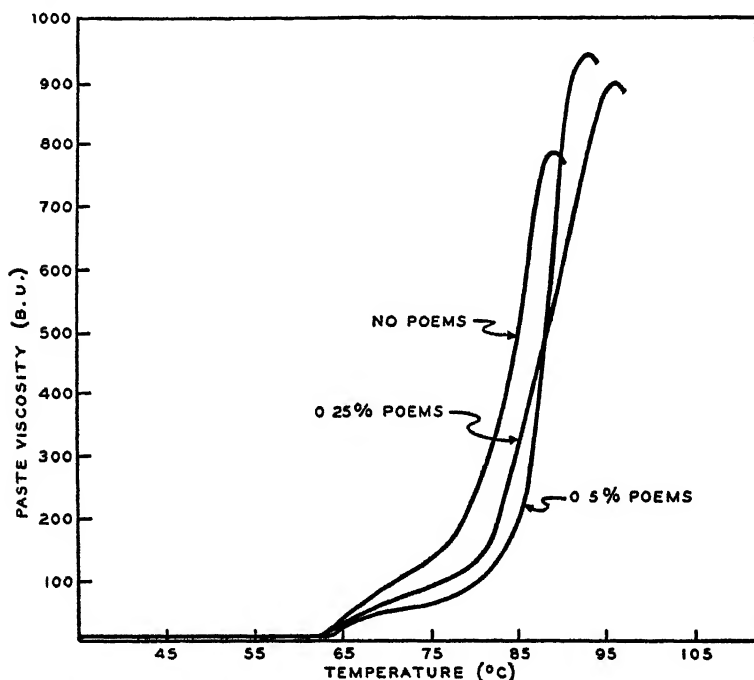


Fig. 3. Effect of polyoxyethylene monostearate on amylograph paste viscosity of a non-diastated flour in the presence of 20 p.p.m. mercuric chloride.

The results (Figs. 2 and 3) suggest that alpha-amylase inactivation was not a major factor, if a factor at all, in the increase in maximum paste viscosity observed earlier; the marked effect in the absence of alpha-amylase indicated a modification of the starch substrate. These curves also indicate that POEMS inhibited granule swelling at temperatures slightly above the transition temperature.

Experiments have shown that POEMS precipitates a portion of the starch from a corn starch gel. Precipitation of this nature may be due to hydrogen bonding of the ester with starch through the terminal hydroxyl group or through the ether oxygens of the polyoxyethylene

chain. Linear organic molecules containing a polar group have long been known to bond with amylose (8) but have not been reported to coordinate with amylopectin. Analogous complex formations are believed to occur between POEMS and starch.

Schoch and French (9) have observed that polar organic compounds possessing both hydrophilic and hydrophobic groupings restrict granule swelling and thereby prevent leaching of the amylose. POEMS has been shown to exert the same effect on the starch of wheat flour in the foregoing studies. This phenomenon may be explained by

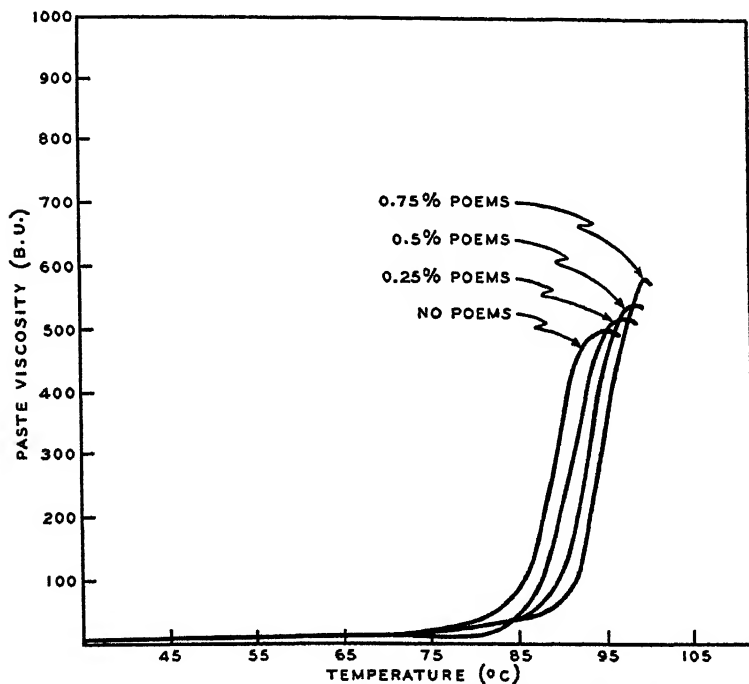


Fig. 4. Effect of polyoxyethylene monostearate on amylograph paste viscosity of commercial corn starch.

assuming the polar group to be oriented with chains of amylose, or possibly with the end branches of amylopectin, near the granule surface. Such an increase in hydrophobic character might well explain the restriction in viscosity of starch pastes at temperatures slightly above the transition temperature. With additional heating in the amylograph, however, the granule may become so distended that the fatty acid chains no longer are able to restrict hydration and a sudden rise in viscosity is manifested. The increase in maximum paste viscosity is possibly caused by an actual increase in granule



diameter through the addition of polyoxyethylene stearate chains; the granule dimensions and mechanical friction are thereby increased. The possibility also exists that restricted distention in the early stages of gelatinization allows less mechanical breakdown of the granule with a consequent higher viscosity in the later stages.

*Effect on Corn Starch Pastes.* Thirty-six grams of commercial corn starch and 450 ml. of water, with and without increments of POEMS, were pasted in the amylograph to determine the effect of the ester on the viscosity of corn starch pastes. The results in Fig. 4 show that POEMS restricted granule swelling in the initial stages of

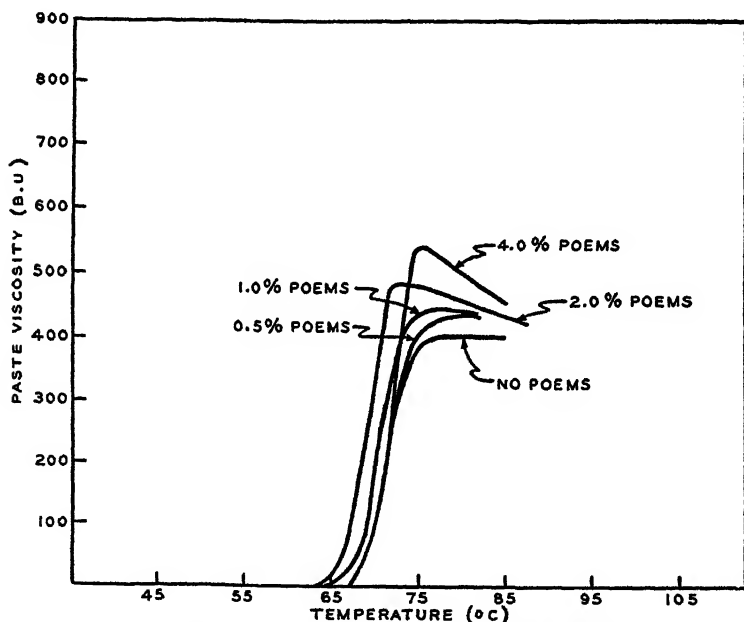


Fig. 5. Effect of polyoxyethylene monostearate on paste viscosity of waxy maize starch.

gelatinization and increased maximum paste viscosity. The magnitude of the increases in corn starch pastes however, were not as great as those observed with wheat flour pastes.

*Effect on Waxy Maize Starch Pastes.* As a means of determining the effect of POEMS on amylopectin, 32 g. of waxy maize starch were suspended in 450 ml. of water and gelatinized in the amylograph. Increments of POEMS were provided by replacing a portion of the water with an equal volume of a 2.5% POEMS suspension.

As is evident from Fig. 5, POEMS increased the maximum paste viscosity of waxy maize starch pastes, but the effect was less pro-

nounced than that produced on either corn starch or wheat flour. Gelatinization temperatures were unaffected, and the inhibition of granule swelling in the early stages observed in the instances of corn and wheat starch, was noticeably absent. These results suggest the possibility that POEMS may bond with amylopectin, but to a lesser extent than with corn or wheat starch. While waxy maize starch contains traces of amylose, this fraction is apparently tightly bound within the granule since a gelatinized waxy maize starch does not produce the blue color with iodine. Response to the iodine test becomes positive only after such a paste is autoclaved. It is not likely that more than trace amounts of amylose are leached out of the granule under the conditions of pasting in the amylograph. While it is doubtful that such large molecules as polyoxyethylene monostearate could exhibit any appreciable amount of mobility within the intricate branching network of amylopectin, the possibility that the outermost branches of amylopectin are susceptible to bonding is not so remote.

*Effects of POEMS on Amyolytic Susceptibility of Starch as Measured by the Modified Wohlgemuth Technique.* The modified Wohlgemuth procedure for the determination of alpha-amylase activity described by Sandstedt, Kneen, and Blish (7) was employed to determine the effect of POEMS on amyolytic dextrinization. This method measures alpha-amylase activity as a function of dextrinization time in the presence of excess beta-amylase.

Increments of POEMS were added to digestion mixtures in the form of 5 ml. aliquots of aqueous POEMS suspensions of appropriate strength. The enzyme source was a 2:5 water extract of barley malt flour.

Results of this experiment demonstrated that this technique could not be used validly in a study of POEMS-starch systems since no end-point was produced which could be regarded as truly achromic with the dextrin standard. Dextrinization time could be determined when POEMS was present in amounts less than 0.25% (based on the weight of soluble starch used in the preparation of the alpha-amylodextrin substrate) and little effect on alpha-amylase activity could be detected at these levels. At higher percentages the dextrin end-point was obscured by the presence of a blue POEMS-amylose-iodine precipitate which was detectable even after 30 hrs. of amyolysis. That POEMS precipitates a portion of the amylose and thus renders it not susceptible, or at least much less susceptible, to amyolytic hydrolysis, is established. It was likewise noted that POEMS precipitated portions of the alpha-amylodextrin substrate during incubation.

If the blue amylose-iodine precipitate be allowed to settle to the bottom of the test tube, the supernatant may be observed as a dextrin

red-brown color and the sample may then be regarded in terms of dextrinization. However, no attempt was made to make a quantitative measure of dextrinization time after settling of the precipitate, since the time involved would depend upon the amount of susceptible substrate remaining, the quantity of which would vary with the percentage of POEMS used.

*Effect of POEMS on Maltose Production.* To determine the effect of POEMS on production of maltose from starch, the following method

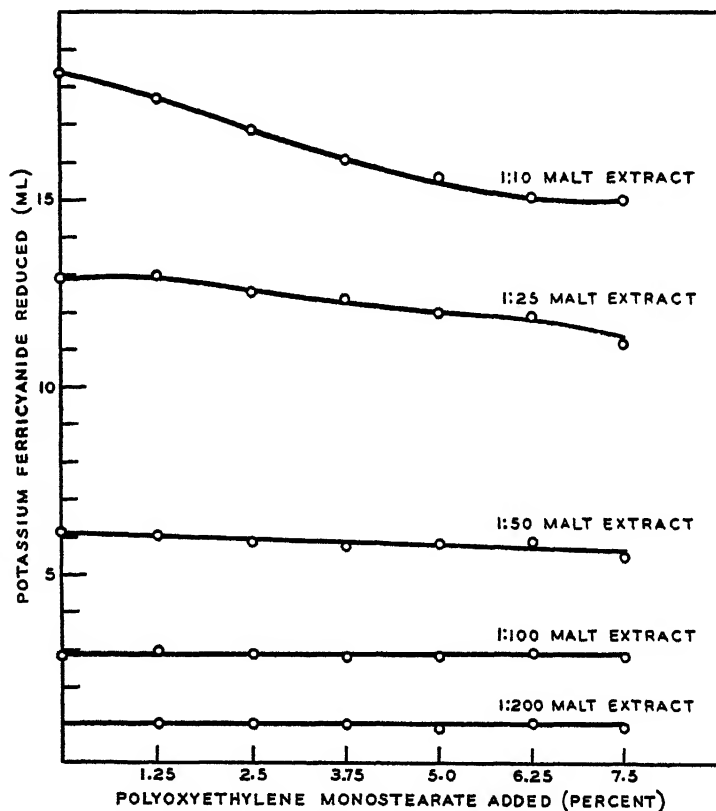


Fig. 6. The effects of polyoxyethylene monostearate on maltose production.

was applied. A mixture of the substances indicated below was incubated for 15 min. at  $30^{\circ}\text{C.} \pm 0.5^{\circ}\text{C.}$ :

|                                                  |            |                    |
|--------------------------------------------------|------------|--------------------|
| (1) Distilled water                              | } to total | 9 ml.              |
| (2) POEMS—indicated increment of 1.0% suspension |            |                    |
| (3) Buffered 2.0% Lintner soluble starch         |            |                    |
| (4) Malt extract—indicated strength              |            |                    |
|                                                  |            | 20 ml.             |
|                                                  |            | 1 ml.              |
|                                                  |            | <hr/> Total 30 ml. |

POEMS was added to the soluble starch immediately before the addition of malt extract. Following the incubation period 10 ml. of 0.5 *N* sodium hydroxide were added to the sample, and a 5 ml. aliquot taken for the determination of reducing sugars. The potassium ferricyanide oxidation procedure as described in *Cereal Laboratory Methods* (1) for measuring diastatic powers of malt was used for determining the maltose produced. Blank tests were made for each series of determinations.

The data given in Fig. 6 demonstrate that POEMS affects maltose production only when present in high percentages in conjunction with very high malt levels. It appears that the observed reduction in maltose produced at high levels of malt and POEMS is not attributable to enzymatic inhibition but rather to a reduction in the quantity of susceptible substrate present. It is concluded from these data that maltose production in fermenting doughs is probably unimpaired by the levels of POEMS commonly employed.

### Discussion

Since the foregoing experiments were preliminary in scope, the results suggest several aspects which warrant future investigation. Without doubt, a study of the effect of POEMS on the individual starch fractions warrants additional experimentation. The affinity of the agent for amylopectin, both in the presence and in the absence of amylose, should be determined. The results of any studies relating the starch fractions to the problem of bread staling must be interpreted in such a way as to allow for the possibility that the firmness retarding mechanism of POEMS involves primarily its action on the entire granule. Whether starch gelatinization presupposes complete rupture of its structure or only extreme distention has been discussed by Schoch (8) who regards the latter viewpoint to be "eminently reasonable." It is doubtful that granules are completely disrupted in the baking of bread, and hence this factor must be considered in any study dealing with the influence of starch fractions.

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## THE INFLUENCE OF DRYING TEMPERATURE AND DRY ICE TREATMENT ON THE QUALITY OF IMMATURE WHEAT <sup>1</sup>

MAX MILNER AND JOHN A. JOHNSON

### ABSTRACT

Drying immature hard red winter wheat from a moisture value of 50 to 14% moisture content at 40 F. caused a reduction in maltose value and in alpha- and beta-amylase activity in comparison with grain dried at room temperature. The alpha-amylase activity of this very immature wheat which was dried at room temperature was much greater than that of similar samples harvested subsequently at lower moisture values and handled in the same manner. A smaller reduction in maltose value occurred when immature grain with 26.5% moisture was dried at 50 F. Dry ice treatment had little effect on several of the biochemical properties of wheat although germination was damaged when grain moisture was high. Loaf volume and crumb grain were affected adversely by dry ice treatment of wheat and these effects appeared to be due to the carbon dioxide rather than to the low temperature in the grain caused by the dry ice.

The influence of harvest conditions and storage treatment on the milling and baking quality of immature or freshly harvested wheat is generally believed to be important, yet meager information on these factors exists. Swanson (5) found that an elevated maltose value was obtained by drying immature wheat in the shock at low temperatures (50°F.). It was desired to re-examine these results and to study other chemical and physical factors which might be affected by drying conditions.

Considerable interest existed during the harvest seasons of 1948 and 1949, particularly in the southwest wheat producing area, concerning the use of dry ice (solid carbon dioxide) in stored grain to destroy insects and decrease grain temperature and respiration. No information was at hand regarding the influence of such treatment on wheat and flour quality and it seemed of interest therefore to initiate studies along this line.

<sup>1</sup> Manuscript received December 27, 1949.

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### Materials and Methods

*Preparation of Wheat Samples.* A plot of hard red winter wheat (Pawnee variety) on the Kansas State College Agronomy Farm was selected. The weather was warm and sunny throughout the filling and ripening period following initiation of harvesting on June 14, 1949, with the exception of one light shower on June 22. The sample harvested by hand on June 14, contained 50% moisture, that taken on June 17, 26.5% moisture and a final sample of fully mature grain harvested by combine on June 24 had a moisture content of 14.0%. On the day prior to the shower on June 22, the moisture content of the wheat in the field was 13.0%.

The following treatments were accorded the various wheat samples.

Sample No. 1. Wheat heads harvested by hand on June 14 when the grain was in the dough stage and contained 50% moisture, were threshed with a small nursery thresher. Two portions of this sample were treated separately as follows: (a) A portion of the fresh sample was spread out immediately to dry on a laboratory bench at about 80°F. with the aid of an electric fan. (b) Immediately after harvest a sample was sealed in a flask and held in a refrigerator at 40°F. for 48 hours. It then was spread out thinly to dry in the refrigerator at this temperature.

Sample No. 2. The grain obtained on June 17 containing 26.5% moisture was dried to 12% moisture on a laboratory bench with the aid of an electric fan. Prior to milling, the wheat was reconditioned to 15% moisture.

Sample No. 3. Same original grain as Sample 2 was spread out thinly for drying in the cold room at 50°F. After five days the grain had a moisture value of 14.0%. Prior to milling, this wheat was reconditioned to 16% moisture.

Sample No. 4. Same original grain as Sample 2, air-dried in the laboratory to 16% moisture and then milled immediately.

Sample No. 5. Same original grain and drying treatment as Sample 4, but before milling, treated with 2% of coarsely granulated dry ice in a tape-sealed can insulated with burlap and cardboard. Sample was held in this manner for 48 hours before the grain was milled. All the following samples which received dry ice treatment were handled in the same manner.

Sample No. 6. Same as Sample No. 5 but treated with 20% of dry ice.

Sample No. 7. Original grain with 26.5% moisture was dried in the laboratory to 12.5% moisture and treated with 2% of dry ice for 48 hours, followed by reconditioning to 16% moisture content prior to milling.

Sample No. 8. Same as Sample 7, but treated with 20% dry ice.

Sample No. 9. Original grain dried as Samples 7 and 8, reconditioned to 16% moisture prior to treatment with 2% dry ice.

Sample No. 10. Same as Sample 9 but treated with 20% dry ice.

Sample No. 11. Original grain (26.5% moisture) was treated with  $\frac{1}{3}$  of its weight in dry ice. The sample then was air-dried for 14 hours to 15% moisture and milled.

Sample No. 12. The original grain was air-dried in the laboratory for 12 hours to a moisture content of 18.5% and then stored in a sealed glass jar which had been flushed with nitrogen, in the laboratory deep freeze unit at  $-20^{\circ}\text{F}$ . The jar was removed after 24 days, and after being warmed to room temperature the grain sample was air-dried to 15% moisture and milled.

Sample No. 13. Fully mature, field-ripened wheat, harvested June 24, with a moisture value of 14%.

*Chemical Determinations on Wheat and Flour.* The following determinations were carried out on the wheat samples in accordance with methods outlined in Cereal Laboratory Methods, 5th edition: (a) moisture content, (b) protein content, (c) ash content, and (d) maltose value.

Saccharifying activity and free and total alpha- and beta-amylase activity were determined by the methods of Kneen and Sandstedt (3) and Kneen, Miller and Sandstedt (2).

Additional determinations on flour included (a) water absorption determined with the farinograph by titration to the 500 unit line, (b) maximum viscosity of flour-water paste in the amylograph using 65 g. of flour and 450 ml. of water, and (c) baking test employing a commercial sponge-dough procedure with and without potassium bromate (Johnson and Miller, 1).

Germination values obtained by the normal procedure with and without pre-chilling the seed were supplied by the Kansas State Seed Laboratory.

Milling was carried out with the Buhler experimental mill. Extraction varied from 68 to 70%.

## Results

*Influence of Drying at Low Temperature on Properties of Immature Wheat.* The analytical results obtained on the very immature wheat (50% moisture) appear in Table I together with data for field-matured wheat harvested later from the same plot.

The very immature wheat was considerably higher in maltose value and in free and total alpha-amylase activity than mature grain. These results confirm those of Schwimmer (4). Drying the immature wheat

TABLE I  
INFLUENCE OF DRYING TEMPERATURE ON MALTOSE VALUE AND AMYLASE  
ACTIVITY OF VERY IMMATURE WHEAT. (WHEAT CONTAINED  
50% MOISTURE AT HARVEST)

| Treatment of wheat        | Maltose value | Saccharifying activity <sup>1</sup> |       | Beta-amylase activity <sup>1</sup> |       | Alpha-amylase activity <sup>2</sup> |       |
|---------------------------|---------------|-------------------------------------|-------|------------------------------------|-------|-------------------------------------|-------|
|                           |               | Free                                | Total | Free                               | Total | Free                                | Total |
| Dried at room temperature | 276           | 0.78                                | 0.82  | 0.73                               | 0.77  | 0.46                                | 0.55  |
| Dried at 40°F.            | 133           | 0.59                                | 0.66  | 0.58                               | 0.65  | 0.05                                | 0.10  |
| Field-matured wheat       | 204           | 0.63                                | 0.70  | 0.63                               | 0.68  | <0.02                               | —     |

<sup>1</sup> Grams starch converted to sugar at 30° C. by 1 g. sample in one hour.

<sup>2</sup> Grams starch dextrinized by 1 g. sample in one hour at 30° C. in presence of excess beta-amylase.

at low temperature significantly reduced the maltose value in comparison with similar wheat dried at room temperature. This latter result is quite opposite to that recorded by Swanson (5) who noted a considerable increase in maltose values when wheat in the shock was dried in a room at 50°F. in comparison with the maltose value of the same grain which had been dried in the sun. Drying at low temperature

TABLE II  
BIOCHEMICAL PROPERTIES OF FRESELY HARVESTED WHEAT AS INFLUENCED  
BY DRYING AND STORAGE AT LOW TEMPERATURE AND  
BY TREATMENT WITH DRY ICE

| Sample no. | Treatment                                          | Maltose value | Germination |           | Sacchari-fying activity <sup>2</sup> |       | Beta-amylase activity <sup>2</sup> |       | Alpha-amylase activity <sup>2</sup> |       |
|------------|----------------------------------------------------|---------------|-------------|-----------|--------------------------------------|-------|------------------------------------|-------|-------------------------------------|-------|
|            |                                                    |               | Reg-ular    | Pre-chill | Free                                 | Total | Free                               | Total | Free                                | Total |
| 2          | Dried to 12% moisture at room temp., reconditioned | 166           | 64          | 70        | 0.62                                 | 0.71  | 0.62                               | 0.70  | <0.02                               | 0.05  |
| 3          | Dried 50°F.                                        | 140           | 66          | 65        | 0.63                                 | 0.69  | 0.63                               | 0.69  | <0.02                               | <0.02 |
| 4          | Dried to 16% moisture                              | 170           | 81          | 90        | 0.64                                 | 0.70  | 0.64                               | 0.69  | <0.02                               | <0.02 |
| 5          | Dried to 16% moisture, 2% dry ice                  | 174           | 62          | 60        | 0.64                                 | —     | 0.64                               | —     | <0.02                               | <0.02 |
| 6          | Dried to 16% moisture, 20% dry ice                 | 172           | 71          | 54        | 0.65                                 | 0.72  | 0.65                               | 0.71  | <0.02                               | <0.02 |
| 7          | Dried, 2% dry ice, reconditioned                   | 170           | 65          | 76        | —                                    | —     | —                                  | —     | —                                   | —     |
| 8          | Dried, 20% dry ice, reconditioned                  | 157           | 61          | 76        | —                                    | —     | —                                  | —     | —                                   | —     |
| 9          | Dried, reconditioned, 2% dry ice                   | —             | —           | —         | —                                    | 0.70  | —                                  | 0.69  | <0.02                               | <0.02 |
| 10         | Dried, reconditioned, 20% dry ice                  | —             | —           | —         | —                                    | 0.69  | —                                  | 0.69  | <0.02                               | <0.02 |
| 11         | Fresh damp grain, 33% dry ice                      | 187           | 20          | 30        | 0.64                                 | 0.70  | 0.64                               | 0.71  | <0.02                               | <0.02 |
| 12         | 18.5% moisture, 24 days at -20°F.                  | —             | 63          | —         | 0.64                                 | 0.69  | 0.63                               | 0.69  | <0.02                               | 0.04  |
| 13         | Field ripened wheat                                | 204           | 91          | 91        | 0.63                                 | 0.70  | 0.63                               | 0.68  | <0.02                               | <0.02 |

<sup>1</sup> Samples 2 to 12 inclusive contained 26.5% moisture at harvest. Sample 13 was field ripened wheat containing 14.0% moisture.

<sup>2</sup> Grams starch converted to sugar at 30°C. by 1 g. sample in one hour.

<sup>3</sup> Grams starch dextrinized by 1 g. sample in one hour at 30°C. in presence of excess beta-amylase.



also reduced the saccharifying activity and alpha- and beta-amylase activity. These same properties remained high in the grain dried at normal temperatures. These values decreased with the normal maturation process, as indicated by the analyses for the normal field-matured wheat harvested at a later date.

*The Properties of Freshly Harvested Wheat and of the Flour and Bread Produced from It, After Dry Ice Treatment.* The effects on a number of wheat properties, of various treatments including drying, storage and dry ice treatment are summarized in Table II.

With one exception, no drastic change in germination or biochemical properties occurred in immature wheat subjected to the wide variety of conditions listed in this table. The maltose value of the sample dried at 50°F. was lower than that of the sample dried at room temperature. This agrees with the similar but larger difference found in the previous experiment when the moisture of the grain at harvest was considerably greater.

The effect of dry ice treatment was remarkably inconsequential in view of the drastically low temperatures to which this immature grain was subjected. Samples containing as much as 16% moisture and treated with dry ice in amounts of 20% or more reached temperatures well below -75°F., yet no deleterious effect on germination or biochemical properties resulted. The one significant effect appeared in the grain which contained 26.5% moisture and which was treated with one-third of its weight of dry ice. In this one case a sharp decrease in germination resulted. A sample of the same wheat containing 18.5% moisture and stored for 24 days in a deep-freeze cabinet at -20°F. showed no apparent decrease in germinative capacity.

Pre-chilling prior to the test for germination generally stimulated germination with immature wheat. The fully mature grain apparently had passed through the dormant period, since the normal germination of 91% was not stimulated by pre-chilling. None of these treatments affected the activity of the amylase enzymes as indicated by the data in the last six columns of Table II.

Several chemical properties of the flours produced from the wheat samples listed in Table II appear in Table III and the biochemical properties are shown in Table IV. The moisture content, protein and ash were with few exceptions fairly consistent in this series of flours, indicating that the milling procedure was fairly uniform. The maltose value (Table IV) tended to decrease when immature wheat was artificially dried. Thus the flour from immature wheat which was dried to 12% moisture in the room and reconditioned before milling (Sample No. 2) had a maltose value of 137, whereas the wheat dried only to 16% moisture and milled, produced flour with a maltose value of 179

TABLE III  
CHEMICAL ANALYSIS OF FLOUR SAMPLES MILLED FROM WHEAT DRIED  
AND STORED AT NORMAL AND LOW TEMPERATURES, AND  
AFTER TREATMENT WITH DRY ICE

| Sample no. | Treatment                                                | Moisture | Protein <sup>1</sup> | Ash <sup>1</sup> |
|------------|----------------------------------------------------------|----------|----------------------|------------------|
|            |                                                          | %        | %                    | %                |
| 2          | Dried to 12% moisture at room temperature, reconditioned | 14.1     | 8.7                  | 0.41             |
| 3          | Dried 50°F.                                              | 14.7     | 8.8                  | 0.40             |
| 4          | Dried to 16% moisture                                    | 14.9     | 8.8                  | 0.47             |
| 5          | Dried to 16% moisture, 2% dry ice                        | 14.5     | 9.4                  | 0.51             |
| 6          | Dried to 16% moisture, 20% dry ice                       | 14.6     | 9.2                  | 0.47             |
| 7          | Dried, 2% dry ice, reconditioned                         | 14.3     | 8.8                  | 0.42             |
| 8          | Dried, 20% dry ice, reconditioned                        | 14.5     | 8.8                  | 0.42             |
| 9          | Dried, reconditioned, 2% dry ice                         | 14.8     | 8.8                  | 0.42             |
| 10         | Dried, reconditioned, 20% dry ice                        | 14.6     | 8.7                  | 0.42             |
| 11         | Fresh damp grain, 33% dry ice                            | 14.2     | 9.1                  | 0.59             |
| 12         | 18.5% moisture, 24 days at -20°F.                        | 13.8     | —                    | —                |
| 13         | Field-ripened wheat                                      | 14.5     | 8.5                  | 0.42             |

<sup>1</sup> 14% moisture basis

(Sample No. 4). The maltose value of flour from wheat dried at low temperature was somewhat lower than that from wheat dried at room temperature (Sample No. 3).

Treatment of wheat with dry ice appeared to have little effect on the maltose value of the flour milled from it (Samples 5 to 11 inclusive). It is notable that the flour obtained from field-ripened wheat (Sample No. 13) had the highest maltose value of all. This wheat, as has been

TABLE IV  
BIOCHEMICAL PROPERTIES OF FLOUR SAMPLES MILLED FROM WHEAT DRIED  
AND STORED AT NORMAL AND LOW TEMPERATURES, AND  
AFTER TREATMENT WITH DRY ICE

| Sample no. | Treatment                                                | Maltose value | Maximum amylograph viscosity | Farinograph absorption <sup>1</sup> | Valorimeter value |
|------------|----------------------------------------------------------|---------------|------------------------------|-------------------------------------|-------------------|
| 2          | Dried to 12% moisture at room temperature, reconditioned | 137           | 800                          | 52.6                                | 46                |
| 3          | Dried 50°F.                                              | 133           | 990                          | 54.7                                | 46                |
| 4          | Dried to 16% moisture                                    | 179           | 600                          | 57.4                                | 45                |
| 5          | Dried to 16% moisture, 2% dry ice                        | 152           | 960                          | 55.5                                | 47                |
| 6          | Dried to 16% moisture, 20% dry ice                       | 130           | 930                          | 53.6                                | 51                |
| 7          | Dried, 2% dry ice, reconditioned                         | 142           | 960                          | 54.3                                | 47                |
| 8          | Dried, 20% dry ice, reconditioned                        | 155           | 920                          | 55.0                                | 46                |
| 9          | Dried, reconditioned, 2% dry ice                         | 130           | 950                          | 53.3                                | 46                |
| 10         | Dried, reconditioned, 20% dry ice                        | 144           | 900                          | 53.6                                | 46                |
| 11         | Fresh damp grain, 33% dry ice                            | 134           | —                            | 51.2                                | 46                |
| 12         | 18.5% moisture, 24 days at -20°F.                        | —             | 960                          | 56.2                                | 48                |
| 13         | Field-ripened wheat                                      | 204           | 865                          | 58.0                                | 44                |

<sup>1</sup> 14% moisture basis.

pointed out, was matured in ideally warm weather with little rainfall of consequence.

Values for amylograph viscosity show considerable uniformity with the exception of two samples. Sample No. 2 had a value of 800 units which was somewhat below the average of the group, while Sample No. 4 milled from wheat which had been dried to 16% moisture but otherwise untreated showed a value of only 600. This value appears anomalous in view of the other normal properties of the wheat and flour. The lowest absorption was exhibited by the flour obtained from the wheat stored in the presence of dry ice with 26.5% moisture (Sample No. 11). Valorimeter values appearing in the table show little variation among the different flours.

TABLE V  
CHARACTERISTICS OF BREAD PRODUCED FROM VARIOUS WHEATS

| Sample no. | Treatment                                                | Loaf volume |               | Crumb grain |               |
|------------|----------------------------------------------------------|-------------|---------------|-------------|---------------|
|            |                                                          | No bromate  | 2 mg. bromate | No bromate  | 2 mg. bromate |
|            |                                                          | c.c.        | c.c.          |             |               |
| 2          | Dried to 12% moisture at room temperature, reconditioned | 2450        | 2625          | 70          | 70            |
| 3          | Dried 50°F.                                              | 2375        | 2500          | 50          | 75            |
| 4          | Dried to 16% moisture                                    | 2250        | 2575          | 60          | 80            |
| 5          | Dried to 16% moisture, 2% dry ice                        | 2250        | 2600          | 50          | 75            |
| 6          | Dried to 16% moisture, 20% dry ice                       | 2200        | 2550          | 50          | 70            |
| 7          | Dried, 2% dry ice, reconditioned                         | 2150        | 2500          | 50          | 75            |
| 8          | Dried, 20% dry ice, reconditioned                        | 2225        | 2500          | 50          | 70            |
| 9          | Dried, reconditioned, 2% dry ice                         | 2225        | 2475          | 50          | 80            |
| 10         | Dried, reconditioned, 20% dry ice                        | 2225        | 2525          | 50          | 75            |
| 11         | Fresh damp grain, 33% dry ice                            | 2250        | 2600          | 50          | 75            |
| 12         | 18.5% moisture, 24 days at -20°F.                        | 2225        | 2425          | 70          | 85            |
| 13         | Field-ripened wheat                                      | 2000        | 2225          | 65          | 80            |

The characteristics of the bread baked from these flours appear in Table V. These data show, surprisingly, that the best loaf volume was produced by the immature wheat dried at room temperature (Sample No. 2), rather than from the field-ripened grain (Sample No. 13). A decrease in loaf volume occurred on drying the immature grain at 50°F. (Sample No. 3) and on milling wheat dried only to 16% moisture prior to milling (Sample No. 4). Dry ice treatment caused a small but significantly uniform reduction in both loaf volume and crumb quality of bread derived from immature wheat (Samples 5 to 11 inclusive). However, loaf volume and internal characteristics were not significantly inferior in the bromated loaves from the dry ice-treated grain when these were compared with the sample dried at room temperature and reconditioned for milling, without preliminary dry ice treatment (Sam-

ple No. 2). It is notable that the field-ripened sample (No. 13) yielded considerably lower loaf volumes from both bromated and unbromated doughs than did the immature wheat regardless of the treatment applied. Sample No. 11 is of particular interest since this was obtained from immature wheat with 26.5% moisture which had been treated with  $\frac{1}{8}$  of its weight of dry ice for 48 hours, and which reached a temperature below  $-75^{\circ}\text{F}$ . The gas-retaining properties of this flour were not harmed to any greater extent than were those of flour from grain treated with dry ice at much lower moisture values, and furthermore the bromate response which it exhibited was excellent. However, the crumb grain of this sample appeared to be damaged to an extent similar to that in the other carbon dioxide-treated samples.

### Discussion

These preliminary results indicate that drying very immature wheat at low temperature ( $40^{\circ}\text{F}$ .) causes a decrease in the alpha- and beta-amylase activity of the flour. This conclusion is supported by the fact that a reduction in maltose value occurred upon drying wheat harvested at 50% moisture and at 26.5% moisture at low temperatures. In the first case this reduction was shown to be accompanied by differences in free and total saccharogenic activity and in free and total alpha- and beta-amylase activities. No explanation is offered now for the marked difference in the results for maltose value obtained here from those of Swanson (5); and further study of this discrepancy is indicated. In this connection, furthermore, it was shown that flour from immature wheat which had been thoroughly dried prior to reconditioning for milling, had a significantly lower maltose value than flour from the same grain dried only to a moisture value of 16% before milling. Partial explanation of this difference may be found in the data of Schwimmer (4) who noted that the solubility of the beta-amylase of immature wheat was irreversibly reduced by drying the grain.

Treatment of immature wheat with dry ice at considerable concentration (2% and 20%) under conditions which allowed the subliming ice to cool the grain to very low temperatures (below  $-75^{\circ}\text{F}$ .) is shown in this study to have little influence on the physical and biochemical properties of the wheat with the exception of a reduction in germination of the sample which contained 26.5% moisture. Experimentation with these high concentrations of dry ice and the use of insulation appear to be justified since, in commercial applications, grain directly contiguous to dry ice would undergo the same treatment accorded in this study to the freshly harvested grain.

The baking study indicated that dry ice treatment caused a reduction in crumb quality as evidenced by coarse and open grain. A small

reduction in loaf volume also occurred due to dry ice treatment, indicating that the gas retention properties of the doughs were affected. That these deleterious effects are not attributable primarily to the low temperature attained by the grain with dry ice treatment, is seen from the fact that this same wheat which had been stored at  $-20^{\circ}\text{F}$ . for 24 days with 18% moisture and without dry ice treatment, showed crumb characteristics at a par with flour from immature wheat which had been dried at room temperature without other treatment. The nature of the biochemical damage caused by dry ice to the grain as manifested by poorer grain and volume characteristics was not indicated in this study. This result raises the possibility that carbon dioxide generated by excessive respiration in immature grain might similarly injure wheat quality.

An interesting point in this study is the fact that immature wheat harvested at 26.5% moisture and dried in the room yielded bread of considerably better loaf volume and crumb grain than the same wheat which had been allowed to ripen in the field under apparently ideal maturing conditions. The data obtained in this study are, however, insufficient to indicate the degree of significance of this fact.

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# FACTORS AFFECTING THE COLOR OF MACARONI.

## III. VARIETAL DIFFERENCES IN THE RATE OF PIGMENT DESTRUCTION DURING MIXING <sup>1</sup>

G. N. IRVINE, C. A. WINKLER, AND J. A. ANDERSON

### ABSTRACT

The rates of pigment destruction during mixing in distilled water, 0.001 *M* cyanide, 30% alcohol, and 0.001 *M* alpha naphthol have been investigated with two series of durum varieties. All varieties behave similarly in the various media. The data clearly indicate the different effects produced by cyanide and alpha naphthol; cyanide inhibits the enzymic reaction, and alpha naphthol acts merely as an anti-oxidant. Good and poor varieties may be characterized by an activity coefficient which is calculated from the rate of oxidation, weighted in terms of the pigment available for oxidation. For a series of 13 durum varieties of widely differing quality, this coefficient gave a good indication of macaroni-making quality. There is good evidence that the enzymic activity is characteristic of a variety, but that it is also influenced by environment.

A prior paper (3) in this series dealt with the reaction responsible for the destruction of the xanthophyll pigments of semolina during mixing. Evidence was reported which indicated that the pigment is oxidized through a coupled reaction simultaneously with the peroxidation of unsaturated fats in the dough by the enzyme lipoxidase. During this work, three major inhibiting agents were found: cyanide ion, alpha naphthol, and alcohol.

The present paper is concerned with two variety studies which were made during the more extensive investigation previously reported. The first study was made primarily to determine whether or not the characteristics of the oxidation reaction in water, cyanide, and 30% alcohol were similar for varieties of differing macaroni-making quality. Quite large samples of material were available and the reaction was investigated over a fairly wide range of mixing times. The second series of varieties was much more extensive than the first but much less material was available. It was felt, however, that the evidence obtained with the first series justified using fewer mixing times to obtain the information that was desired from the second series. This series provided a better opportunity to investigate the extent to which rate of oxidation

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is a varietal characteristic and also to contrast the behavior of cyanide and alpha naphthol as inhibiting agents.

### Materials and Methods

The first series of varieties comprised the named varieties Carleton, Mindum, Pelissier, and Golden Ball, and two plant breeder's hybrids designated as Bald Medeah  $\times$  1317 and 1742. Each variety represented a composite made up from material grown at the same group of experimental stations in Western Canada. But two of the composites represented the 1945 crop and four the 1946 crop.

The second series contained most of the durum varieties which have, at one time or another, been grown in Western Canada; namely, the standard variety, Mindum, the recent varieties Carleton and Stewart, one of the old red durum varieties Pentad, and nine other varieties (see Table III). Each variety sample was composited from material grown in 1946 at 11 experimental stations across Canada. This series was grown for special tests by the Dominion Laboratory of Cereal Breeding and only small samples could be spared for this investigation.

The material in both experiments was processed, as outlined in a previous paper (3), by the disc method of Cunningham and Anderson (2); 31% absorption was used for samples mixed with distilled water, cyanide or alpha naphthol solutions, and 33% for samples mixed with 30% alcohol. Pigment content was determined on the ground discs by the standard method (1) employing the butyl alcohol solvent in conjunction with an Evelyn colorimeter.

### Results and Discussion

*Series I.* Samples were mixed for intervals from one to ten minutes in distilled water, 0.001 *N* potassium cyanide, and 30% alcohol; the reaction curves are shown in Fig. 1. In general, the varieties all yielded similarly shaped curves for a particular treatment. In distilled water, all varieties with the exception of Carleton yielded a three-stage curve. The Carleton sample exhibited an induction period between the first and second stages which previous evidence (3) has shown to be an absorption effect; studies of other samples of Carleton have shown that this behavior is not typical of this variety, but is probably typical of all varieties at some particular absorption level depending on the sample. With 0.001 *N* cyanide, all varieties exhibited a three-segment curve, five out of six showing the slight reversal of the reaction during the third stage. With 30% alcohol the curves show only two segments; for Carleton the slope of the second segment is slightly negative, and for two of the other varieties there is also some reversal at the longer mixing times.

Discussion of the results will be limited to the first two segments of the reaction curves as it is improbable that the reaction ever proceeds beyond this stage during macaroni processing. The rapid initial portion of the curve has been called the "initial reaction," the following

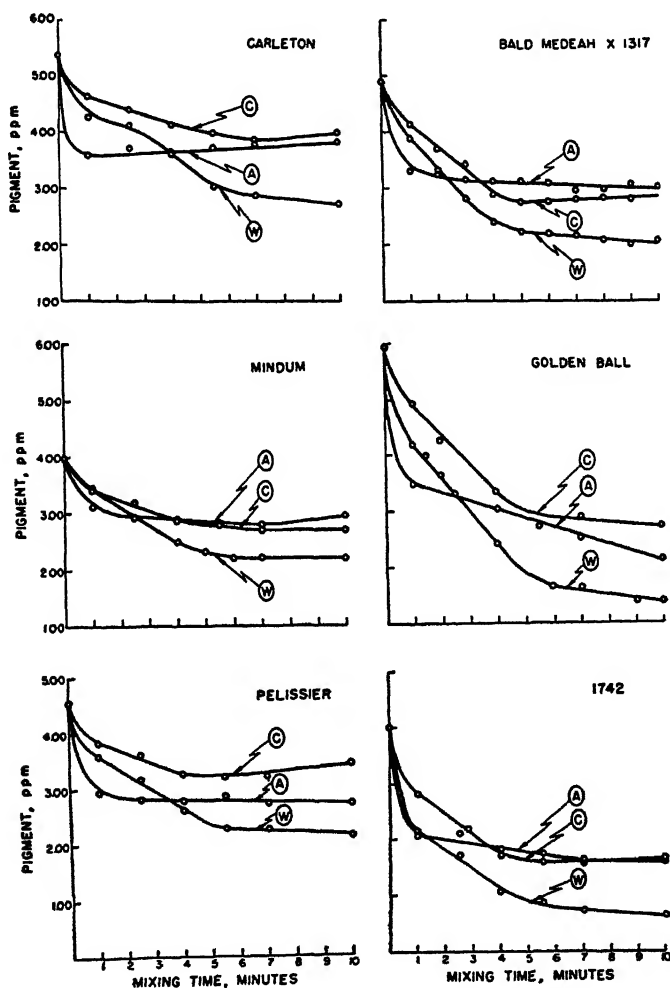


FIG. 1. Loss of pigment during mixing in distilled water (W), 0.001 *N* cyanide (C), and 30% alcohol (A) for six varieties of durum wheat.

zero order segment, the "mixing reaction." Table I gives the rate of the initial reaction for each sample in each of the three media as determined from the reaction curves; the percentage inhibition by cyanide and the percentage inhibition or acceleration by alcohol are calculated from these rates.



Comparison of the initial pigment concentration with the rate of the initial reaction (Table I) shows that the rate of reaction is largely independent of pigment level. The rate of the initial reaction varies over a considerable range—from 0.61 to 1.87 p.p.m. per minute, a factor of three; for all but one sample, which is unaffected, cyanide inhibits this rate from 24 to 42%. For five samples out of six, alcohol accelerates the rate, in some cases as much as 66%; one sample is unaffected.

Table II lists data for the mixing reaction. The rate of the mixing reaction is largely independent of pigment level and varies, less widely than the rate of the initial reaction for the samples studied, from 0.235 to 0.510 p.p.m. per minute, a factor of slightly more than two. Cyanide inhibits the rate of the mixing reaction for five of the six varieties as much as 53%; overall inhibition of the reaction to ten minutes mixing by cyanide is marked for all of the varieties tested, and varies from

TABLE I  
INHIBITION OF THE INITIAL REACTION BY 0.001 *M*  
CYANIDE AND BY 30% ALCOHOL

| Variety        | Initial pigment<br>p.p.m. | Rate of initial reaction<br>p.p.m. per minute |         |         | Inhibition of initial<br>reaction, % |         |
|----------------|---------------------------|-----------------------------------------------|---------|---------|--------------------------------------|---------|
|                |                           | Water                                         | Cyanide | Alcohol | Cyanide                              | Alcohol |
| Mindum         | 4.02                      | 0.61                                          | 0.62    | 0.90    | 0                                    | 48*     |
| Pelissier      | 4.57                      | 0.97                                          | 0.73    | 1.61    | 25                                   | 66*     |
| Carleton       | 5.39                      | 1.11                                          | 0.75    | 1.82    | 32                                   | 64*     |
| Golden Ball    | 5.98                      | 1.80                                          | 1.04    | 2.49    | 42                                   | 38*     |
| 1742           | 4.02                      | 1.87                                          | 1.20    | 1.88    | 36                                   | 0       |
| B. Med. × 1317 | 4.94                      | 1.11                                          | 0.84    | 1.63    | 24                                   | 47*     |
| Mean           | 4.90                      | 1.25                                          | 0.85    | 1.61    | 32                                   | 29*     |

\* Percentage acceleration.

27 to 52%. Inhibition of the mixing reaction by 30% alcohol is very marked in all cases, ranging from 67 to 100%, and the net balance between acceleration of the initial reaction and inhibition of the mixing reaction after ten minutes of mixing shows an overall inhibition in all cases, varying from 17 to 60%.

This experiment indicates two things. Firstly, the characteristics of the oxidation reaction in each of the three media are, in general, the same for durum varieties of widely differing macaroni-making quality; the differences in quality show up essentially as differences in the rates of various phases of the reaction. Secondly, it is evident, from a comparison of the rates of reaction in cyanide and 30% alcohol that although both media effect an overall inhibition of the reaction, the mechanism of inhibition is quite different. This difference has been discussed in a previous paper (3).

TABLE II  
INHIBITION OF THE MIXING REACTION BY 0.001 *M*  
CYANIDE AND BY 30% ALCOHOL

| Variety        | Initial pigment p.p.m. | Rate of mixing reaction p.p.m. per minute |         |         | Inhibition of mixing reaction, % |         | Overall inhibition, % |         |
|----------------|------------------------|-------------------------------------------|---------|---------|----------------------------------|---------|-----------------------|---------|
|                |                        | Water                                     | Cyanide | Alcohol | Cyanide                          | Alcohol | Cyanide               | Alcohol |
| Mindum         | 4.02                   | 0.310                                     | 0.147   | 0.062   | 53                               | 80      | 27                    | 41      |
| Pelissier      | 4.57                   | 0.310                                     | 0.150   | 0.015   | 52                               | 95      | 52                    | 23      |
| Carleton       | 5.39                   | 0.300                                     | 0.147   | 0.033   | 51                               | 100     | 48                    | 41      |
| Golden Ball    | 5.98                   | 0.501                                     | 0.527   | 0.150   | 5*                               | 70      | 29                    | 17      |
| 1742           | 4.02                   | 0.330                                     | 0.369   | 0.095   | 12*                              | 74      | 31                    | 29      |
| B. Med. X 1317 | 4.94                   | 0.500                                     | 0.400   | 0.040   | 20                               | 92      | 30                    | 32      |
| Mean           | 4.90                   | 0.365                                     | 0.284   | 0.071   | 22                               | 80      | 37                    | 35      |

\* Percentage acceleration.

*Series II.* These samples were processed at two mixing times, one minute and four minutes, with distilled water, 0.01 *N* cyanide, and 0.001 *M* alpha naphthol. Rates of the initial and mixing reactions were calculated from pigment losses for zero to one minute and for one to four minutes. The rates of the *initial* reactions in the three media, and inhibition as per cent and in terms of its pigment equivalent, for cyanide and alpha naphthol, are given in Table III; the rate of the *mixing* reaction in the three media, with the percentage inhibition by cyanide and alpha naphthol, the overall inhibition, and a series of activity coefficients, are given in Table IV.

TABLE III  
INHIBITION OF INITIAL REACTION BY 0.01 *N* CYANIDE  
AND 0.001 *M* ALPHA NAPHTHOL

| Variety     | Initial pigment (p.p.m.) | Rate of initial reaction (p.p.m. per minute) |         |                    | Inhibition % |                    | Pigment equivalent of inhibition (p.p.m.) |                    |
|-------------|--------------------------|----------------------------------------------|---------|--------------------|--------------|--------------------|-------------------------------------------|--------------------|
|             |                          | Water                                        | Cyanide | $\alpha$ -naphthol | Cyanide      | $\alpha$ -naphthol | Cyanide                                   | $\alpha$ -naphthol |
| Carleton    | 4.91                     | 0.82                                         | 0.56    | 0.00               | 32           | 100                | 0.26                                      | 0.82               |
| Mindum      | 4.40                     | 0.82                                         | 0.39    | 0.00               | 52           | 100                | 0.43                                      | 0.82               |
| Goose       | 4.20                     | 0.63                                         | 0.38    | 0.00               | 40           | 100                | 0.25                                      | 0.63               |
| Arnautka    | 3.70                     | 0.72                                         | 0.72    | 0.13               | 0            | 82                 | 0.00                                      | 0.59               |
| Stewart     | 4.92                     | 0.89                                         | 0.43    | 0.15               | 52           | 83                 | 0.46                                      | 0.74               |
| Kubanka     | 4.14                     | 1.13                                         | 0.55    | 0.24               | 51           | 79                 | 0.58                                      | 0.89               |
| Kahla       | 4.91                     | 1.14                                         | 0.75    | 0.22               | 34           | 81                 | 0.39                                      | 0.92               |
| Pelissier   | 4.99                     | 1.37                                         | 0.78    | 0.45               | 43           | 67                 | 0.59                                      | 0.92               |
| Acme        | 2.86                     | 0.66                                         | 0.36    | 0.06               | 45           | 91                 | 0.30                                      | 0.60               |
| Monad       | 3.32                     | 0.77                                         | 0.46    | 0.00               | 40           | 100                | 0.31                                      | 0.77               |
| Pentad      | 3.29                     | 0.84                                         | 0.55    | 0.21               | 35           | 75                 | 0.29                                      | 0.63               |
| Bald Medeah | 4.17                     | 1.12                                         | 0.96    | 0.43               | 16           | 61                 | 0.18                                      | 0.69               |
| Golden Ball | 4.51                     | 1.71                                         | 1.54    | 0.91               | 10           | 47                 | 0.17                                      | 0.80               |

With these data, certain relationships between each phase of the reaction and the various factors have been examined and are discussed below.

*The Initial Reaction.* A plot of the initial pigment concentration against the rate of the initial reaction indicated that these factors are largely independent; for eight of the thirteen varieties the rates were approximately the same, while for the remaining five the rates were somewhat higher but did not reflect the initial pigment concentration. The rate in cyanide followed the rate in water to some extent, the correlation coefficient being significant beyond the 1% point; however, this was due almost entirely to the effect of the same five varieties which showed a higher rate in water. The rate in alpha naphthol also followed the rate in water, the correlation being highly significant and again, due largely to the same five varieties.

Comparison of the percentage inhibition of the initial reaction by cyanide and alpha naphthol clearly illustrates the difference between the action of these two inhibiting agents; cyanide inhibition varies from 0 to 52% (average value 35%) while alpha naphthol inhibition varies from 47 to 100% (average value 82%). It was suggested in the previous paper (3) that cyanide acted on an activating system rather than directly on the enzyme; this would reduce the effectiveness of the enzymic oxidation but should not reduce it to zero. Alpha naphthol, on the other hand, acts as an antioxidant and thus should largely prevent oxidation of the pigment until the added alpha naphthol is exhausted. This difference is shown more clearly by comparing the pigment equivalents of the alpha naphthol and cyanide inhibition, these vary from 0.59 to 0.92 p.p.m. (mean, 0.76 p.p.m.) for alpha naphthol, and from 0.00 to 0.59 p.p.m. (mean, 0.32 p.p.m.) for cyanide. Although the rate in water varies threefold, from 0.63 to 1.71 p.p.m. per minute, the pigment equivalent of the alpha naphthol inhibition is roughly constant about the mean value of 0.76 p.p.m. In contrast, a plot of the cyanide equivalent against the rate in water shows a wide random scatter with no apparent trend.

*The Mixing Reaction.* A plot of the initial pigment concentration against the rate of the mixing reaction indicated that there is no relation between these two factors. The rate of the mixing reaction followed the rate of the initial reaction reasonably well; the correlation between these two was 0.87 ( $1\% = 0.68$ ). This would be expected on the basis of the hypothesis proposed for the mechanism of the reaction (3) if all samples had been milled and stored under similar conditions.

For ten of the thirteen samples, the rate in cyanide followed the rate in water very closely; two varieties, Golden Ball and Stewart,

appeared to be more sensitive to cyanide, while Kubanka was less so. These differences did not appear during the initial reaction.

The rate in alpha naphthol appears to be largely independent of the rate in water.

Inhibition by cyanide is more pronounced during the mixing reaction than during the initial reaction, varying from 20 to 100% (average 52%). This behavior contrasts with that of alpha naphthol which affects the mixing reaction much less than the initial reaction, inhibition varying from 59% to three cases of slight acceleration (average 14%); it appears that the amount of alpha naphthol added just prevents the initial reaction but is exhausted early in the mixing reaction. This

TABLE IV  
INHIBITION OF MIXING REACTION BY 0.01 *N* CYANIDE  
AND BY 0.001 *M* ALPHA NAPHTHOL

| Variety     | Rate of mixing reaction<br>(p.p.m. per minute) |         |                    | Inhibition<br>% |                    | Overall inhibition<br>% |                    | Activity<br>coefficient |
|-------------|------------------------------------------------|---------|--------------------|-----------------|--------------------|-------------------------|--------------------|-------------------------|
|             | Water                                          | Cyanide | $\alpha$ -naphthol | Cyanide         | $\alpha$ -naphthol | Cyanide                 | $\alpha$ -naphthol |                         |
| Carleton    | 0.17                                           | 0.07    | 0.17               | 59              | 0                  | 44                      | 66                 | 0.13                    |
| Mindum      | 0.17                                           | 0.06    | 0.15               | 65              | 12                 | 58                      | 69                 | 0.14                    |
| Goose       | 0.18                                           | 0.09    | 0.10               | 50              | 49                 | 45                      | 76                 | 0.15                    |
| Arnautka    | 0.16                                           | 0.06    | 0.16               | 63              | 0                  | 26                      | 50                 | 0.16                    |
| Stewart     | 0.27                                           | 0.00    | 0.11               | 100             | 59                 | 74                      | 73                 | 0.20                    |
| Kubanka     | 0.20                                           | 0.16    | 0.25               | 20              | *                  | 40                      | 42                 | 0.20                    |
| Kahla       | 0.27                                           | 0.18    | 0.37               | 33              | *                  | 35                      | 33                 | 0.22                    |
| Pelissier   | 0.31                                           | 0.20    | 0.25               | 35              | 19                 | 39                      | 48                 | 0.26                    |
| Acme        | 0.20                                           | 0.09    | 0.18               | 55              | 10                 | 50                      | 53                 | 0.27                    |
| Monad       | 0.24                                           | 0.14    | 0.27               | 42              | *                  | 40                      | 45                 | 0.29                    |
| Pentad      | 0.23                                           | 0.12    | 0.24               | 48              | 0                  | 41                      | 39                 | 0.29                    |
| Bald Medeah | 0.31                                           | 0.20    | 0.24               | 36              | 23                 | 24                      | 44                 | 0.31                    |
| Golden Ball | 0.44                                           | 0.16    | 0.39               | 64              | 11                 | 34                      | 31                 | 0.48                    |

\* Slight acceleration.

contrast provides additional support for the hypothesis suggested to account for the behavior of these inhibiting agents.

From the data on overall inhibition, it is clear that *in general* cyanide has a marked inhibiting effect on the reaction responsible for pigment destruction and that the same is true of alpha naphthol.

*The Activity Coefficient.* The activity coefficient (last column of Table IV) is obtained by dividing the pigment destroyed in the interval from one to four minutes of the mixing reaction by the amount remaining at the end of the initial reaction (after one minute). It appears to be a useful measure of the potential contribution of the yellow pigment factor to macaroni quality. No suggestion is made that this figure gives a reliable index of macaroni-making quality since it takes no account of the brown pigment, and therefore, gives a reasonable meas-

ure of relative macaroni-making quality only when the amount of brown pigment is small. The claim for the activity coefficient is that it indicates relatively how much pigment will remain after a given treatment, and provides a useful index of enzymic activity.

In spite of the large differences in initial pigment content among the varieties studied, the coefficient gives an excellent indication of the enzymic activity as well as a reasonable indication of the amount of pigment remaining after four minutes of mixing. The correlation between activity coefficient and pigment content after four minutes mixing, is 0.80 ( $1\% = 0.68$ ). For this series, then, in which environmental effects were largely eliminated, the activity coefficient is characteristic of the varieties and could be very useful in distinguishing between good and bad varieties. It is interesting to compare these activity coefficients with others calculated for different samples of some of these varieties during the course of this whole investigation:

|                 |      |                  |      |
|-----------------|------|------------------|------|
| Pelissier       | 0.23 | Carleton         | 0.13 |
| (variation 22%) | 0.28 | (variation 8%)   | 0.13 |
|                 | 0.26 |                  | 0.14 |
| Golden Ball     | 0.49 | Mindum           | 0.27 |
| (variation 18%) | 0.42 | (variation 240%) | 0.34 |
|                 | 0.48 |                  | 0.10 |
|                 |      |                  | 0.14 |

These data emphasize a point which has been noted for many years in testing plant breeders' varieties: Mindum is a rather unstable variety, being as good as the best varieties in some years, but being considerably poorer in others. Although no similar data on the variation of the activity coefficient are available for Stewart, experience with other varietal series has indicated that this variety is also more unstable than Carleton, although not to as marked a degree as Mindum. It is also interesting to note the effect of mixing good and poor varieties. Although the data at present are very limited, it has been found that a mixture of about 50% Golden Ball with 50% of the best varieties yields semolina which has an activity coefficient of 0.35 and gives a reaction curve typical of Golden Ball alone. A more extensive investigation of this phenomenon is planned for the near future.

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## STUDIES WITH DOGS FED FLOUR TREATED WITH AMMONIUM PERSULFATE<sup>1</sup>

AARON ARNOLD and FRANS C. GOBLE

### ABSTRACT

Dogs were fed diets which contained high levels of untreated flour or of flour treated with 0.15% or 1.0% ammonium persulfate for periods of time up to 16 months. Observations were made throughout of gross appearance, weight gains, kidney dye clearances, blood picture (hemoglobin, total red and white cell counts, and differential white cell count including the polymorphonuclear neutrophiles, eosinophiles, lymphocytes, and monocytes), followed by examination of the thoracic and abdominal viscera, the endocrines, the central nervous system at autopsy, and microscopic study of the organs. On the basis of these studies it is concluded that ammonium persulfate, even in amounts which are more than 200 times those which would be present in commercially treated flour, is an innocuous ingredient in the diet.

As a result of Mellanby's report (8) on the toxicity of nitrogen trichloride-treated flour, great interest has been focused on possible undesirable effects of other agents used for treating flour. As indicated in a recent review (1), several investigators have shown that only nitrogen trichloride, of all agents thus far tested in short-term feeding trials, exerts any deleterious action.

This communication gives the results obtained on dogs when ammonium persulfate was used. Reports (6) indicate that this agent is used widely in Europe and at one time most of the flour in England was treated with it. Tests with dogs are practically mandatory in view of the fact that observations with this species first brought to light an unfavorable effect due to the use of nitrogen trichloride for flour treatment. Also, dogs have proven to be the species most sensitive to the effects of the toxic agent produced by the nitrogen trichloride treatment of flour.

### Materials and Methods

*Diets.* The composition of the diets, patterned after those of Mellanby (8), was given in detail in a previous paper (2). In addition to two dogs fed control diets, six dogs were fed a diet which contained flour with 0.15% or 68 g. of ammonium persulfate per cwt (45.4 kg.), and four dogs were fed a diet which contained flour with 1.0% or 454 g. of ammonium persulfate per cwt (45.4 kg.).

<sup>1</sup> Manuscript received February 23, 1950.

Contribution from Sterling-Winthrop Research Institute, Rensselaer, N. Y.

The flour and yeast components of the diet were steamed 90 minutes before feeding. Thus, the persulfate exerted its action upon the flour prior to its ingestion. As observed by Kent-Jones and Amos (6), tests (4) indicated no free persulfate at the end of the steaming period. The end product of persulfate appears to be ammonium sulfate, an agent routinely added to bread doughs as a yeast food. Diets containing nitrogen trichloride-treated flour which had been subjected to steaming in the same way were manifestly toxic to dogs (8).

*Dogs.* In all, 12 dogs were used in this study. Seven of them had previously not been used for any tests. However, the tests which are given below on five of them, numbers 9, 10, 12, 15, and 17, represent further study on dogs earlier used and reported (2). In that study, the dogs had been subjected to several successive short-term feeding trials of diets which contained one or another of several flour maturing agents. Thus, five of the dogs had experienced serious untoward reactions due to the ingestion of nitrogen trichloride treated flour prior to their use in this portion of the study.

*Organ Function Tests.* To provide additional and more refined methods of confirming the apparent well-being of the dogs, several tests, such as are used for humans in clinics, were performed at approximately monthly intervals on the dogs.

The test (9) for kidney function was based on measuring the capacity of the kidney to clear phenolsulfonphthalein from the bloodstream. The dye was injected intramuscularly into the dogs and the amount present in urine samples taken by catheterization at  $\frac{1}{2}$  and 1 hour after injection was estimated colorimetrically and compared with the amounts injected. Ten readings were obtained on the dogs carried through the entire experimental period.

Supplementary to this, six observations were made during the latter 12 months of test on the amount of urinary protein precipitable by phosphotungstic acid (10) as another measure of possible renal damage. In general, only minute amounts of protein, 0.2g per l of urine, are consistently expected.

*Hematological Tests.* The 17 hematological examinations of the dogs performed at approximately monthly intervals in all cases comprised the following: Total red and white cell counts, hemoglobin content, and differential white cell count. The normal values for each of the above items are known from numerous studies reported in the literature, but collateral studies on control dogs in our own laboratories have been made throughout the course of these investigations.

*Post-mortem Examinations.* Five of the dogs were sacrificed by rapid intravenous injection of Evipal Sodium rather early in the tests (after 22 weeks of test for four of the dogs and six weeks of test for the

control dog). The remaining dogs were sacrificed at the termination of the studies after approximately 16 months of test. A gross examination of the thoracic and abdominal viscera, the endocrines, and the central nervous system was made. Material from the various organs was fixed in 10% formalin for subsequent histological examination. Paraffin sections of the following organs, stained with hemalum and eosin, were examined microscopically: heart, lungs, liver, spleen,

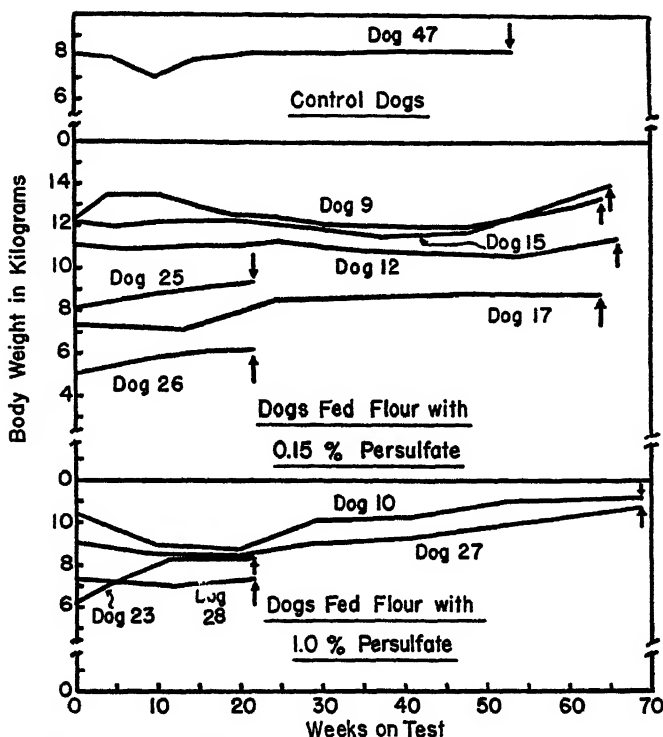


Fig. 1. Weight records of dogs fed diets containing flour treated with none, 0.15%, or 1.0% ammonium persulfate. The arrows indicate the times at which the dogs were sacrificed. Dogs 47, 25, 26, 23, 27 and 28 were fed the indicated diets without any premedication control period. Dogs 9, 10, 12, 15, and 17 were fed the indicated diets subsequent to a 90 day feeding trial of essentially the same regimen except that the flour had been treated with 0.033% ammonium persulfate (2).

kidneys, pancreas, stomach, intestine, lymph nodes, thyroid, adrenal, pituitary, brain (including cerebral cortex, basal ganglia, hippocampus, mammillary bodies, cerebellum, pons), spinal cord, and gonads. In addition, sections of the central nervous system were stained by the following methods for the demonstration of particular structures: Mallory's (7) phosphotungstic acid hematoxylin for glia fibers, cresyl violet for Nissl bodies, Bodian's protargol for axis cylinders and Weil's (11) method for myelin sheaths.



## Results

*Weight Records.* It will be noted from Fig. 1 that the dogs were fed the test diets for 16 months. For five of the dogs, this portion of the study was subsequent to the part reported earlier (2) so that, in all, these five were on test for two years. While a diet high in flour is not particularly well suited to dogs, it is apparent that they were maintained satisfactorily on it. The presence or absence of persulfate treatment obviously did not affect their weights even after extended periods. In addition, the weights of the control dogs did not appear

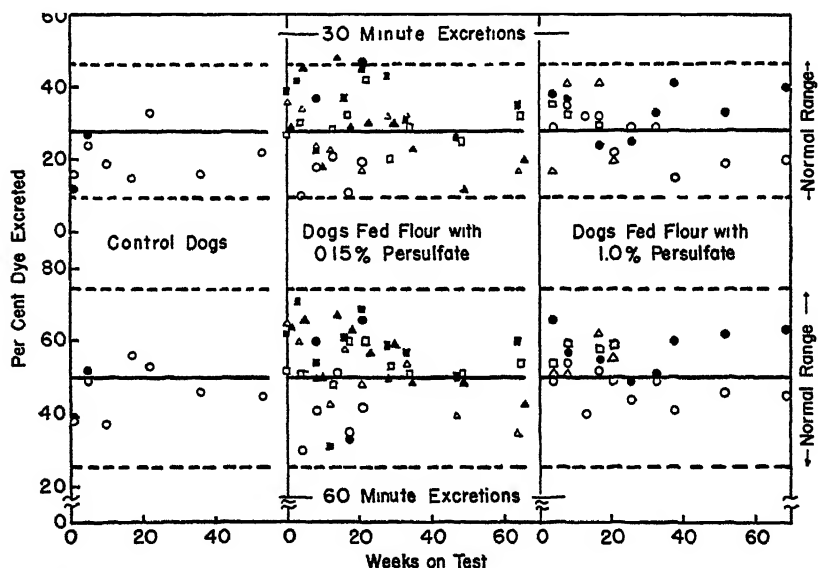


Fig. 2. Kidney function test by dye excretion of dogs fed flour containing diets. Solid lines are average values for normal animals and dotted lines are upper and lower limits ( $\pm 2$  standard deviations) of normal values in our colony as determined in more than 50 dogs. Key to dog numbers: Control dogs—open circle (○) 47, solid circle (●) 48; Dogs fed flour with 0.15% persulfate—open triangle (△) 9, solid triangle (▲) 12, open square (□) 15, solid square (■) 17, open circle (○) 25, solid circle (●) 26; Dogs fed flour with 1.0% persulfate—open circle (○) 10, open triangle (△) 23, solid circle (●) 27, open square (□) 28.

to differ materially from the test dogs, further indicating a lack of difference in the nutritive value of the diets.

*Kidney Function Evaluated by Dye Excretion.* The means  $\pm 2$  s.d. of the kidney function results observed on over 50 normal dogs in our colony have been indicated for reference. The mean excretion at 30 minutes was  $28 \pm 9.2\%$  and, at 60 minutes  $50 \pm 12.2\%$  (means  $\pm$  s.d.).

From the data given in Fig. 2 it may be seen that the excretions fall within the normal range as here defined. The two high  $\frac{1}{2}$  hour values observed early in the test on two of the dogs cannot be regarded

TABLE I  
KIDNEY FUNCTION EVALUATED BY URINARY PROTEIN OUTPUT

[illegible]

TABLE II

SUMMARY (MEANS  $\pm$  S.E.) OF HEMATOLOGICAL OBSERVATIONS ON THE DOGS FED DIETS WHICH SUPPLIED EITHER UNTREATED FLOUR OR FLOUR TREATED WITH 0.15% OR 1.0% OF AMMONIUM PERSULFATE

| Dietary persulfate level | Dog no          | No. readings | Hemo-globin       | Red blood cells $\times 10^6$ | White blood cells $\times 10^3$ | Neutro-philic cells $\times 10^3$ | Eosino-philic cells $\times 10^3$ | Lympho-cytes $\times 10^3$ | Mono-cytes $\times 10^3$ |
|--------------------------|-----------------|--------------|-------------------|-------------------------------|---------------------------------|-----------------------------------|-----------------------------------|----------------------------|--------------------------|
| %                        |                 |              | mg. %             |                               |                                 |                                   |                                   |                            |                          |
| None                     | 47              | 12           | 13.2<br>$\pm .3$  | 6.7<br>$\pm .4$               | 20.4<br>$\pm 1.3$               | 14.2<br>$\pm 1.4$                 | 1.3<br>$\pm .2$                   | 3.9<br>$\pm .4$            | 0.5<br>$\pm .1$          |
| "                        | 31 <sup>1</sup> | 4            | 13.7<br>$\pm 4.5$ | 6.7<br>$\pm .3$               | 10.8<br>$\pm 1.0$               | 7.0<br>$\pm .3$                   | 0.6<br>$\pm .1$                   | 2.8<br>$\pm .2$            | 0.3<br>$\pm .1$          |
| "                        | 32 <sup>1</sup> | 4            | 14.8<br>$\pm 2.2$ | 6.1<br>$\pm .2$               | 10.9<br>$\pm 1.9$               | 6.1<br>$\pm 1.0$                  | .5<br>$\pm .2$                    | 2.0<br>$\pm .4$            | .2<br>$\pm .1$           |
| "                        | 36 <sup>1</sup> | 4            | 17.8<br>$\pm .2$  | 7.3<br>$\pm .1$               | 11.2<br>$\pm 1.5$               | 7.5<br>$\pm .9$                   | 0.5<br>$\pm .1$                   | 2.8<br>$\pm .7$            | 0.4<br>$\pm .1$          |
| "                        | 38 <sup>1</sup> | 4            | 14.8<br>$\pm .2$  | 5.9<br>$\pm .1$               | 19.5<br>$\pm 3.6$               | 13.0<br>$\pm 3.3$                 | 1.1<br>$\pm .3$                   | 4.6<br>$\pm .8$            | 0.7<br>$\pm .2$          |
| "                        | 40 <sup>1</sup> | 4            | 16.4<br>$\pm .4$  | 6.2<br>$\pm .1$               | 15.2<br>$\pm 2.7$               | 8.0<br>$\pm 1.2$                  | 2.8<br>$\pm .7$                   | 4.0<br>$\pm .9$            | .5<br>$\pm .1$           |
| "                        | 43 <sup>1</sup> | 4            | 18.7<br>$\pm .6$  | 7.8<br>$\pm .1$               | 10.1<br>$\pm 1.5$               | 7.2<br>$\pm 1.3$                  | 0.3<br>$\pm .1$                   | 1.9<br>$\pm .2$            | 0.6<br>$\pm .2$          |
| 0.15                     | 9               | 17           | 16.0<br>$\pm .3$  | 6.8<br>$\pm .2$               | 13.0<br>$\pm 1.1$               | 6.9<br>$\pm .8$                   | 1.2<br>$\pm .2$                   | 4.6<br>$\pm .4$            | 0.3<br>$\pm .1$          |
| "                        | 12              | 17           | 14.8<br>$\pm .3$  | 6.9<br>$\pm .2$               | 17.7<br>$\pm 1.1$               | 11.1<br>$\pm .8$                  | 1.0<br>$\pm .1$                   | 5.2<br>$\pm .4$            | 0.4<br>$\pm .1$          |
| "                        | 15              | 17           | 16.0<br>$\pm .4$  | 7.1<br>$\pm .3$               | 11.6<br>$\pm 1.1$               | 6.2<br>$\pm .8$                   | 1.5<br>$\pm .2$                   | 3.7<br>$\pm .3$            | 0.4<br>$\pm .1$          |
| "                        | 17              | 17           | 15.4<br>$\pm .2$  | 8.0<br>$\pm .3$               | 14.1<br>$\pm .7$                | 8.6<br>$\pm .6$                   | 1.3<br>$\pm .5$                   | 5.1<br>$\pm .4$            | 0.5<br>$\pm .1$          |
| "                        | 25              | 5            | 13.3<br>$\pm .4$  | 5.4<br>$\pm .3$               | 15.2<br>$\pm 2.8$               | 9.1<br>$\pm 2.4$                  | 0.7<br>$\pm .1$                   | 5.1<br>$\pm 1.0$           | 0.3<br>$\pm .1$          |
| "                        | 26              | 5            | 14.3<br>$\pm .2$  | 6.7<br>$\pm .4$               | 11.3<br>$\pm 2.2$               | 8.0<br>$\pm 1.0$                  | 0.6<br>$\pm .1$                   | 2.3<br>$\pm .3$            | 0.4<br>$\pm .1$          |
| 1.0                      | 10              | 17           | 16.1<br>$\pm .2$  | 7.3<br>$\pm .3$               | 12.7<br>$\pm .7$                | 8.4<br>$\pm .5$                   | 0.7<br>$\pm .1$                   | 3.4<br>$\pm .2$            | 0.3<br>$\pm .1$          |
| "                        | 23              | 5            | 16.3<br>$\pm .5$  | 6.3<br>$\pm .5$               | 13.5<br>$\pm 2.0$               | 8.7<br>$\pm 1.5$                  | .6<br>$\pm .2$                    | 3.8<br>$\pm .8$            | .2<br>$\pm .1$           |
| "                        | 27              | 17           | 17.4<br>$\pm .3$  | 7.5<br>$\pm .3$               | 12.3<br>$\pm .7$                | 8.2<br>$\pm .5$                   | 0.4<br>$\pm .2$                   | 3.3<br>$\pm .2$            | 0.5<br>$\pm .1$          |
| "                        | 28              | 5            | 14.5<br>$\pm .8$  | 6.2<br>$\pm .3$               | 12.0<br>$\pm 2.3$               | 9.5<br>$\pm 1.8$                  | 0.1<br>$\pm .1$                   | 2.4<br>$\pm .6$            | 0.2<br>$\pm .1$          |

<sup>1</sup> Hematological readings on six control dogs which were not otherwise associated with this study.

as significant evidence of poor function in view of the normal values on the same dogs subsequently.

*Kidney Function Evaluated by Urinary Protein.* The data on the urinary protein excretions are given in Table I. It may be seen that there are no consistent trends of progressive increase of urinary protein output and that the means of the three groups overlap so that no evidence of kidney derangement can be ascribed to any of the dietary treatments. Whether catheterization of the dogs in taking the samples contributed to the wide range of values obtained cannot be indicated with certainty, though it is believed that this may have influenced the results to some extent.

*Urinary Calculi.* The control urine samples were examined for sediment. The samples were by no means free of casts, bacterial cells, "triple phosphates", etc. but these were not present to a marked extent in any of the dogs, nor were any of the three groups of dogs distinguished by any differences. Also, consistent progressive increases in urinary sediment in the dogs could not be established. Here again, irritation due to catheterization probably affected the findings.

*Hematological Examinations.* The hematological findings were calculated so as to yield an individual value for each dog (mean  $\pm$  s.e.). For added comparative purposes, the readings on six control dogs, in no way connected with these studies, have been included in Table II.

It may be noted that, in the elements given in the table, there are no trends which can be correlated with the ingestion of ammonium persulfate treated flour. There is as much variation among the dogs within a group as among the dogs between groups. Thus, this method of following the well-being of the animals disclosed no adverse findings.

*Post-mortem Examinations.* Neither gross nor microscopic examination of the organs of the dogs on test revealed any tissue changes attributable to the ingestion of the ration which included ammonium persulfate treated flour. No conditions were found which did not also occur in the control dogs fed untreated flour. Parenthetically, it may be noted that a careful study did not disclose any lesions in sections of the tissues of two dogs sacrificed while showing symptoms of running fits induced by nitrogen trichloride treated flour.

## Discussion

Under conditions of test which were far more rigorous than those which lead to running fits in dogs fed nitrogen trichloride treated flour, dogs fed ammonium persulfate treated flour show no ill effects. Thus, dogs fed nitrogen trichloride treated flour at commercial levels of treatment (8) developed running fits in a matter of two to four weeks. In contrast, dogs fed ammonium persulfate treated flour for periods up

to 16 months at levels which were approximately 200 times the level of treatment required to achieve maturing action evidenced no adverse reaction as judged by weight maintenance, kidney dye clearance, urinary protein outputs, hematological examinations, post-mortem examinations, or by tissue section study. These observations are in agreement with our own earlier preliminary studies (2) and those of Bentley *et al.* (5).

These observations are in accord, also, with results on rats fed flour containing ammonium persulfate and bread baked from flour treated with ammonium persulfate (3). And finally, this agent has been used extensively on flour intended for human consumption in Europe as indicated by Kent-Jones and Amos (6).

On the basis of the studies summarized here we may conclude that ammonium persulfate is an innocuous ingredient when used for the maturing of flour.

#### Acknowledgments

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## BREAD LOAF VOLUME AND PROTEIN CONTENT OF HARD RED SPRING WHEATS<sup>1</sup>

C. C. FIFIELD,<sup>2</sup> RAY WEAVER,<sup>3</sup> AND J. F. HAYES<sup>3</sup>

### ABSTRACT

The relation between loaf volume and flour protein is reported for 589 samples representing ten varieties of hard red spring wheat grown for four crop years under a wide range of climate and soil conditions. A rich formula containing milk solids, shortening, and potassium bromate in conjunction with optimum mixing time and water requirement was employed in the bread-baking tests.

The relation between loaf volume and flour protein for each variety was linear between the limits of protein encountered, approximately 8.5 to 18.0%. The regression lines for loaf volume on protein content for any variety were similar for the years 1944, 1945, 1946, and 1947, indicating that the bread-baking quality of each variety was essentially the same in different years.

The level and slope of the regression of loaf volume on protein content for the varieties differed significantly, indicating differences between varieties in protein quality.

It is well known that early investigations of the relationship between protein content of wheat and bread loaf volume by Thomas (12), Shollenberger (11) and others indicated a curvilinear relation. Bailey and Sherwood (1) calculated the formula for the curve based on the data of five crop years, 1921-1925.

Zinn (13) in a study of the relationship of the different characters of American grown wheats based on published data (1907 to 1919) obtained high correlation coefficients between loaf volume and protein content. Larmour (8) found with samples of Canadian hard red spring wheats from the 1929 crop that the regression of loaf volume on protein was linear between the limits of 7.0% and 19.3% when a blend-bromate formula was used. Using baking techniques that more fully reflect the potentialities of each flour, linear relations within varieties have since been demonstrated by Barmore, Finney and McCluggage (2, 3) Larmour, Working and Ofelt (9), and Bayfield and West (4). Barmore, Finney and McCluggage (3) and McCalla (10) showed that the level and slope of the regression lines are different for different varieties. Exceptions to this linear relation may occur as a result of

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deleterious effect of excessive high temperature between heading and ripening of the grain on loaf volumes, as shown by Finney and Fryer (7).

If it can be shown that the relation between protein content and loaf volume is generally linear for all classes of wheat, the characterization and evaluation of varieties will be greatly facilitated. This will be true especially for samples that differ in protein content because of inherent differences or environmental conditions. The purpose of this paper is to present additional data bearing on the relation between protein content and loaf volume in hard red spring wheat varieties.

### Materials and Methods

This paper presents results for 589 samples representing ten varieties of hard red spring wheat grown in experimental plots at approximately 25 stations in nine states in the years 1944, 1945, 1946, and 1947. All the samples were grown in the hard red spring wheat region but environmental conditions differed widely between some of the experiment stations. The varieties were Thatcher, Pilot, Rival, Regent, Mida, Ceres, Cadet, Newthatch, Henry, and Pilot  $\times$  Mida No. 1756 (C. I. No. 12303). The number of samples of each variety tested each year varied from six for the variety Henry to 25 for Thatcher.

The samples used in this study were grown under variable but, in general, rather favorable seasonal conditions. The 1944 growing season was perhaps the best of the four. Although seedlings were late the rainfall was abundant, resulting in good yields. The quality of the grain was good, and protein content was about average. The seasons in 1945 and 1946 were late and cool and except for some stem and leaf rust damage the yields were normal and the grain was of good quality. The protein content was high in both of these years with 1946 averaging higher. Excessive rain and cold weather delayed planting of the 1947 crop. The moisture conditions during the growing season were generally good except in northwestern North Dakota and northern Montana where there was a deficiency in moisture. There appears to have been no damage to quality because of high temperature such as that reported by Finney and Fryer (7). The grain was of generally good quality and averaged about the same in protein content as the samples of the 1944 crop.

The samples were milled to 90 per cent patent flours on the Allis-Chalmers experimental flour mill. The flours ranged from 8.5 to 18.0% in protein content and 0.41 to 0.53% in ash content (14.0% moisture basis). The bread-baking tests were made by a formula described by Fifield, *et al.* (5) using 100 grams of flour, 2.0 grams of compressed yeast, 1.5 grams of salt, 5.0 grams sugar, 0.25 grams of malted wheat flour, 3.0 grams of shortening, 4.0 grams of non-

fat dry milk solids, and varying amounts (0 to 4 mg.) of potassium bromate. The ingredients for two loaves of bread were mixed for a sufficient length of time for optimum dough development in a Hobart-Swanson dough mixer with four pins in the head and two pins in the bowl and operated at 108 r.p.m. The doughs after mixing were divided into two equal parts, fermented for 3 hours at 86°F. (30°C.), panned and proofed for 55 minutes at 86°F. (30°C.) then baked for 25 minutes at 450°F. Loaf volumes and other data were averaged for the two loaves. Loaf volume data are presented only for those loaves containing that amount of potassium bromate that produced maximum loaf volumes. In most instances the loaf having the greatest volume also had the best grain, texture, and crumb color. This procedure is substantially the same as that used by Finney and Barmore (6).

### Results and Discussion

*Comparison of Interannual Results Within Variety.* The number of samples, correlation coefficients, and regression equations for bread loaf volume vs. protein content of flour for each variety in each of the years 1944 to 1947 are given in Table I. The relations between loaf volume and flour protein content are shown graphically by means of regression lines in Fig. 1.

The relation between loaf volume and protein content was essentially linear within each variety, as shown in Fig. 1. It is evident from these coefficients ( $r$  varies from + 0.77 to + 0.99), which were all found to be significant at the 1% level, that protein content accounts for a large part of the variation in loaf volume within a variety regardless of season.

The protein content of the samples of each variety in this study with the exception of Henry, ranged from approximately 8.5 to 18.0% depending upon the environment under which they were grown. The protein content for Henry varied from 9.0 to 14.0%. There were fewer high protein samples of Henry, because it was not grown at the stations in the western part of the region where high protein samples of other varieties are generally produced.

The slopes of the regression lines (Fig. 1) for any individual variety were similar for each of the four years. None were significantly different as shown by covariance analysis. The greatest variation appears to be for the variety Henry between the 1946 and 1947 seasons, probably due to the relatively small number of samples for this variety. The agreement between the regression lines for different years seems to be best for Pilot, Cadet, Newthatch, Regent, and Mida. The data indicate that the increment of increase in loaf volume per unit increase



TABLE I  
ANNUAL CORRELATION COEFFICIENTS AND REGRESSION EQUATIONS FOR LOAF VOLUME AND FLOUR PROTEIN  
FOR 10 VARIETIES OF HARD RED SPRING WHEAT

| Variety                 | Number of samples |      |      |      | Correlation coefficient <sup>1</sup> |      |      |      | Regression equation<br>$\hat{Y} =$ |            |            |            |
|-------------------------|-------------------|------|------|------|--------------------------------------|------|------|------|------------------------------------|------------|------------|------------|
|                         | 1944              | 1945 | 1946 | 1947 | 1944                                 | 1945 | 1946 | 1947 | 1944                               | 1945       | 1946       | 1947       |
| Year                    | 14                | 18   | 19   | 19   | 0.88                                 | 0.86 | 0.81 | 0.80 | 190+49.8 X                         | 301+40.9 X | 243+44.4 X | 256+44.7 X |
| Newthatch               | 6                 | 9    | 11   | 9    | 0.97                                 | 0.99 | 0.90 | 0.96 | 116+54.0 X                         | 117+54.4 X | 349+39.0 X | 195+49.2 X |
| Ceres                   | 14                | 18   | 19   | 19   | 0.90                                 | 0.91 | 0.85 | 0.93 | 199+52.4 X                         | 301+43.5 X | 212+50.0 X | 267+45.3 X |
| Cadet                   | 18                | 23   | 25   | 24   | 0.94                                 | 0.91 | 0.86 | 0.89 | 3.8+65.4 X                         | 223+48.8 X | 246+45.8 X | 218+49.3 X |
| Thatcher                | 10                | 12   | 14   | 15   | 0.97                                 | 0.91 | 0.89 | 0.87 | 98+59.4 X                          | 214+50.8 X | 132+56.6 X | 295+44.1 X |
| Regent                  | 14                | 18   | 21   | 20   | 0.95                                 | 0.85 | 0.83 | 0.77 | 112+56.6 X                         | 228+47.7 X | 215+46.9 X | 254+42.9 X |
| Mida                    | 10                | 11   | 14   | 15   | 0.94                                 | 0.80 | 0.90 | 0.87 | 56+65.5 X                          | 88+59.9 X  | 16+64.3 X  | 91+58.8 X  |
| Rival                   | 14                | 19   | 20   | 20   | 0.93                                 | 0.93 | 0.85 | 0.89 | 208+52.4 X                         | 135+58.8 X | 273+46.4 X | 167+54.4 X |
| Pilot                   | 7                 | 13   | 12   | 14   | 0.94                                 | 0.95 | 0.89 | 0.89 | 243+45.3 X                         | 245+46.8 X | 205+47.8 X | 62+58.7 X  |
| Pilot X Mida<br>(N1756) | 6                 | 6    | 10   | 9    | 0.97                                 | 0.87 | 0.82 | 0.93 | 280+47.6 X                         | 441+34.4 X | 178+54.2 X | 47+66.7 X  |
| Henry                   |                   |      |      |      |                                      |      |      |      |                                    |            |            |            |

<sup>1</sup> All correlation coefficients are significant at the 1% level.

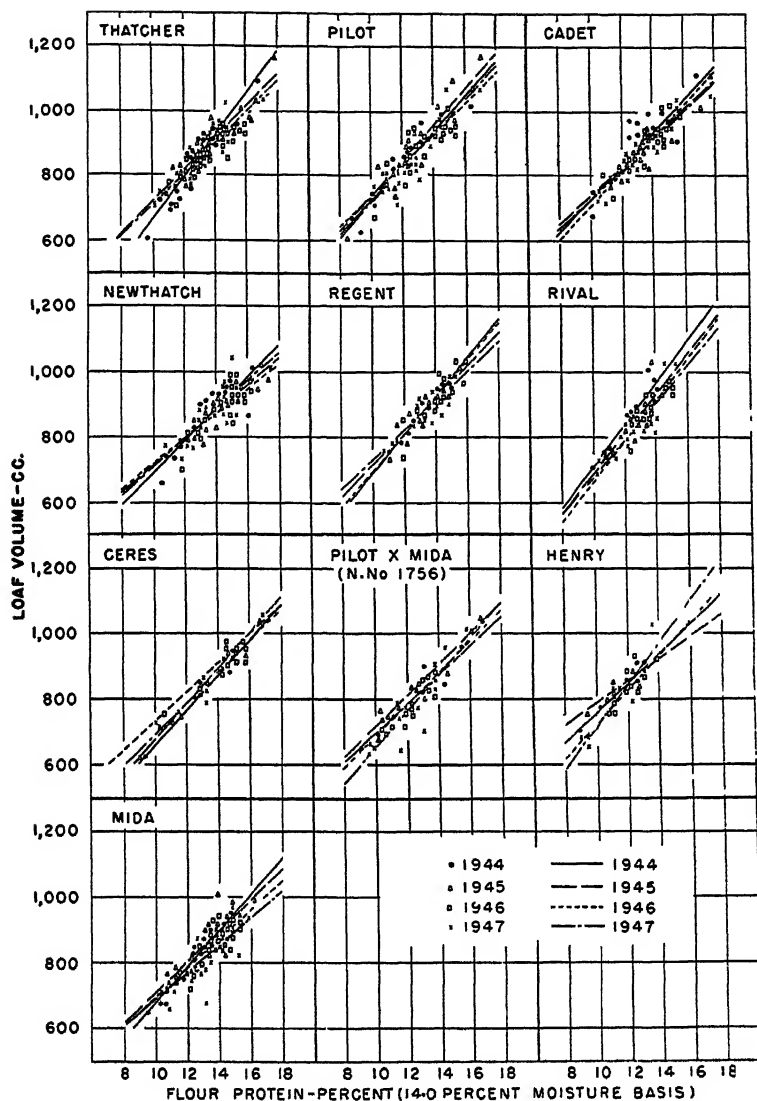


FIG. 1. Annual regression lines for loaf volume and flour protein for ten hard red spring wheat varieties grown for the 1944-1945-1946 and 1947 seasons.

in protein content is essentially the same for any one variety in different years.

*Comparison of Varieties.* Since the regression lines for the same variety are substantially the same for different seasons, a comparison of varieties based on the average of all seasons seems justified. The four-year average protein content, loaf volume, regression equations, and

the loaf volume calculated to 10.0, 12.0, and 15.0% protein for the ten varieties are shown in Table II. The regression lines showing the relation between loaf volume and flour protein content are presented graphically in Fig. 2. It should be noted that all varieties were not grown at all stations and, therefore, their average flour protein contents and loaf volumes are not directly comparable. For example, Henry as noted above was not grown at the western stations in the region where protein contents of the other varieties were high.

The correlation coefficients ( $r$ ) varied from + 0.84 for Newthatch and Mida to + 0.96 for Ceres and were all highly significant. This means that flour protein content accounted for about 17 to 92% of the variation in loaf volume within varieties. The change in loaf

TABLE II  
SUMMARY OF PROTEIN CONTENT AND LOAF VOLUME DATA FOR  
10 VARIETIES OF HARD RED SPRING WHEAT

| Variety      | Number of samples | Average                    |             | Loaf volume at <sup>2</sup> |               |               | Correlation coefficient | Regression equation $\hat{y} =$ |
|--------------|-------------------|----------------------------|-------------|-----------------------------|---------------|---------------|-------------------------|---------------------------------|
|              |                   | Flour protein <sup>1</sup> | Loaf volume | 10.0% protein               | 12.0% protein | 15.0% protein |                         |                                 |
|              |                   | <i>per cent</i>            | <i>cc.</i>  | <i>cc.</i>                  | <i>cc.</i>    | <i>cc.</i>    | ( $r$ )                 |                                 |
| Henry        | 31                | 11.5                       | 820         | 741                         | 846           | 1005          | 0.88                    | 213+52.8 X                      |
| Pilot        | 73                | 12.7                       | 869         | 725                         | 832           | 993           | 0.91                    | 188+53.7 X                      |
| Regent       | 51                | 13.4                       | 892         | 716                         | 820           | 975           | 0.91                    | 197+51.9 X                      |
| Cadet        | 70                | 13.5                       | 889         | 725                         | 819           | 959           | 0.90                    | 257+46.8 X                      |
| Rival        | 50                | 12.9                       | 859         | 678                         | 803           | 991           | 0.86                    | 52+62.6 X                       |
| Thatcher     | 90                | 13.5                       | 874         | 693                         | 797           | 952           | 0.90                    | 175+51.8 X                      |
| Newthatch    | 70                | 14.1                       | 880         | 701                         | 789           | 920           | 0.84                    | 262+43.9 X                      |
| Mida         | 73                | 13.1                       | 835         | 691                         | 784           | 924           | 0.84                    | 225+46.6 X                      |
| Pilot X Mida | 46                | 12.4                       | 803         | 680                         | 783           | 937           | 0.91                    | 165+51.5 X                      |
| Ceres        | 35                | 13.7                       | 865         | 676                         | 779           | 932           | 0.96                    | 163+51.3 X                      |

<sup>1</sup> 14.0% moisture basis.

<sup>2</sup> Calculated from regression equation.

volume for each per cent increase in protein content based on the regression equation varied from 43.9 cc. for Newthatch to 62.6 cc. for Rival. A majority of the variety regression lines, however, had slopes of 50.0 to 56.0 cc. An analysis of covariance, showed that only the slope of the line for Rival differed significantly from those of other varieties. It can be observed in Fig. 2 that high protein flours of Rival produced relatively large loaves of bread; whereas, low protein flours of this variety generally made smaller loaves than did flours of Thatcher having comparable protein content.

Significant loaf volume differences exist between certain of the hard spring varieties at the various protein levels. For example, at 12.0% protein the loaf volume for Henry was 846 cc. in contrast to 779 cc. for Ceres and 783 cc. for Pilot X Mida. Each of the more

important loaf volume differences at 12.0% protein was compared with the corresponding standard error of the difference. In general, a loaf volume difference of about 45 cc. between two varieties was significant at the 5% level; whereas, a difference of more than 60 cc. was significant at or beyond the 1% level. The loaf volume differences between Henry and the varieties Ceres, Pilot  $\times$  Mida, and Mida were 2.91, 3.10, and 3.77 times, respectively, the standard errors of the difference for the paired comparisons, and were significant

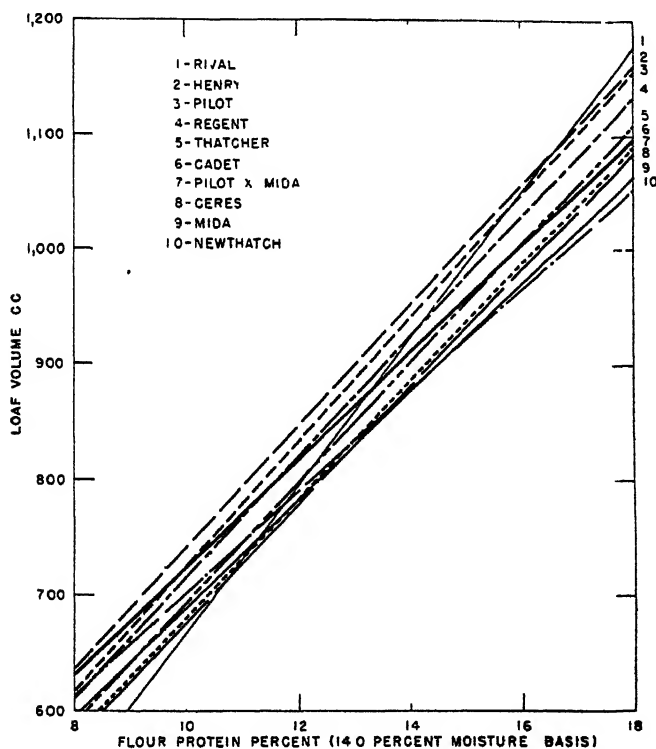


FIG. 2. Loaf volume-flour protein content regression lines for ten varieties of hard red spring wheat grown for four years at several stations.

beyond the 1% level. The average loaf volume differences of about 50 cc. between Pilot and the varieties Ceres and Pilot  $\times$  Mida were found to be significant at the 5% level. The difference of 41 cc. between Regent and Ceres approached significance  $t = 1.94$ . Significance at the 5% level would require that  $t = 1.99$ .

Studies of this laboratory and of Finney and Barmore (6) indicate that regression lines for loaf volume on protein content are reliable indexes of gluten quality and the potential breadmaking value of vari-

eties of wheat. Loaf volume, of course, depends both on the quality and the quantity of the protein in the flour as well as on certain other variable factors.

### Acknowledgments

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# THE CYSTINE CONTENT OF WHEAT FLOUR IN RELATION TO DOUGH PROPERTIES<sup>1</sup>

B. WÖSTMANN<sup>2</sup>

## ABSTRACT

The cystine content of wheat flour proteins determined polarographically varied from 2.2 to 4.5%. The physical properties of salt-water doughs, as reflected by the surface area of the extensograms for 23 flours taken after a rest-time of 135 minutes are positively correlated with protein content, the cystine content of the flour proteins, and the total cystine content of the flour. The correlation coefficients between the extensogram area and these respective variables were +0.82, +0.77, and +0.92.

The disulfide bond has long been regarded as an important structural unit in the protein molecule (8) and several research workers have pointed out the importance of this linkage in determining the properties of the wheat flour proteins (1, 13, 16, 17).

As part of an investigation of the factors which determine the baking quality of flour, the cystine contents of protein preparations from 23 wheat flours were determined by a polarographic method and the results correlated with the physical properties of salt-water doughs, as determined by means of the Brabender Extensograph. The polarographic technique was used to determine cystine because the results are not materially influenced by the carbohydrates which are present in the protein preparations used for analysis.

## Materials and Methods

*Wheat Flours.* For this study 23 wheat flours were employed; seven of these were hard wheat flours and three were soft wheat flours imported from the United States in 1946 and 1947 by the Dutch government. The remainder were soft flours of 80% extraction milled from Dutch wheat. As far as is known, none was bleached.

*Polarographic Determination of Cystine in Flour Protein Hydrolysates.* Cystine was determined by a polarographic method based on that described by Brdicka (6) in 1934. He showed that in 0.1 *N* ammonium hydroxide — 0.1 *N* ammonium chloride as supporting electrolyte, cystine gives a characteristic current-voltage curve in the polarograph if 0.001 *N* cobaltous chloride is added. The cystine is reduced to cysteine at -0.4 volts at the dropping mercury electrode and then

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reacts with cobaltous ions to form a complex at the mercury cathode. Starting at  $-1.5$  volts this complex catalyzes an evolution of hydrogen at the cathode interface, the amount of which is dependent upon the concentration of the complex on the cathode. As long as there is a surplus of cobaltous ions with respect to the amount of cystine present, the concentration of the complex on the cathode surface will be determined by the amount of cystine diffusing to the cathode.

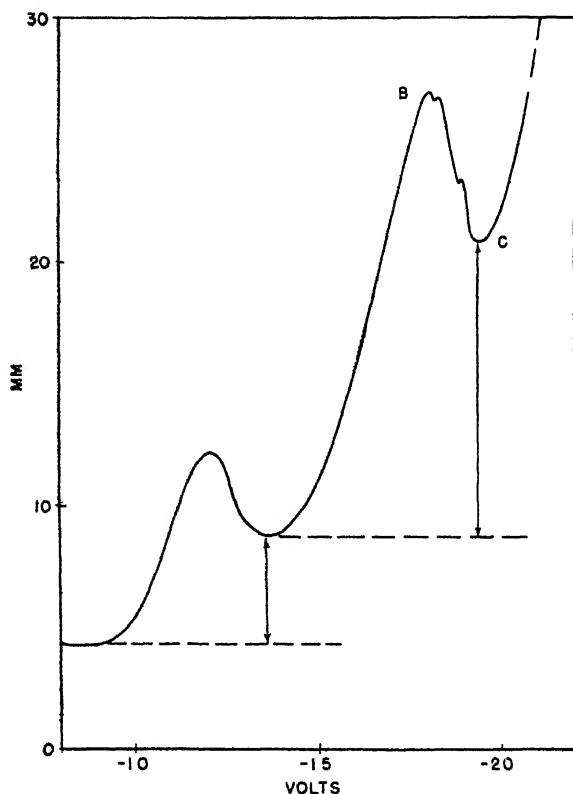


FIG 1 Polarogram of cystine At  $-1.4$  volts wave given by  $\text{Co}^{++}$  ions at  $-1.9$  volts minimum of cystine wave

As a result of the catalytic evolution of hydrogen on the cathode interface which starts at a potential of  $-1.5$  volts, the current-voltage curve assumes the configuration shown in Fig. 1. The potentials are measured against the saturated calomel electrode.

Brdicka took the height of the minimum, C, as a measure of the cystine concentration. In later publications, however, he occasionally used the maximum, B, (7, 10). He pointed out that at concentrations between  $2 \times 10^{-6}$  and  $10 \times 10^{-6}$  moles cystine per liter, the minimum

height is not directly proportional to the cystine concentration, the increase in the height being relatively less as the concentration of cystine increases. Moreover, Sladek and Lipschutz (14) showed that the wave heights for cystine were depressed by the presence of other amino acids. For these reasons Stern, Beach, and Mac (15) employed a special calibration technique for determining the cystine content of protein hydrolysates. Increments of a standard cystine solution were

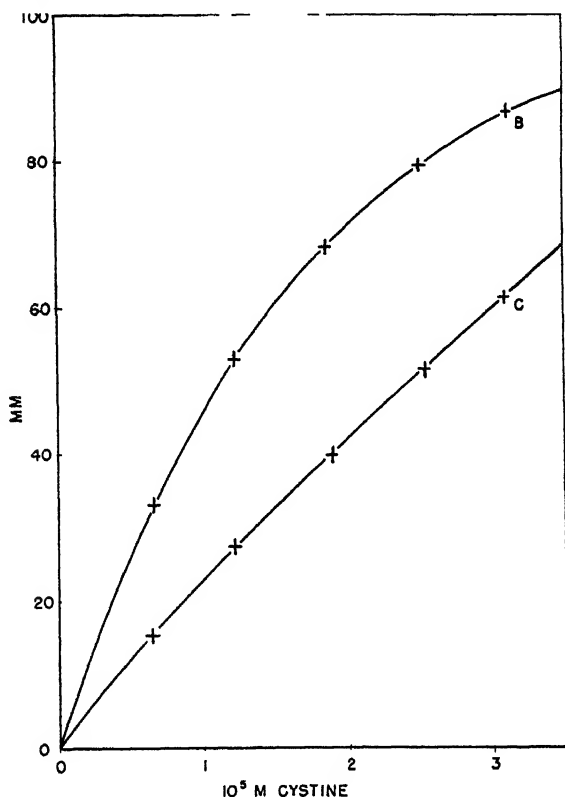


FIG. 2 Height of the maximum B and of the minimum C of the cystine wave as a function of the cystine concentration

added to a given volume of each hydrolysate under study and the relative changes in the height of the polarographic wave were measured. By this method, calibration curves were obtained which applied to the estimation of cystine in that particular hydrolysate. They reported that this technique permitted determining cystine in solutions containing only 12 to 24  $\mu\text{g}/\text{ml}$ . with an accuracy of  $\pm 5\%$ .

Using a drop time of one second and an undamped galvanometer period of 2.5 seconds, a somewhat different conclusion was reached in



the present study. As long as the concentration did not rise above  $4 \times 10^{-3}$  moles cystine per liter, the height at the minimum remained almost directly proportional to the concentration as shown in Fig. 2. If different quantities of cystine were added to a protein hydrolysate, this relation was absolutely linear, provided the total concentration remained below the maximum cited above.

For this reason, it was thought justifiable to add an amount of cystine to the hydrolysate equal to the amount estimated to be present and then calculate the cystine content from the minimum of the curve. In practice, 0.1 mg. cysteine hydrochloride (Hoffmann-LaRoche) was always added to 25 ml. of the solution to be examined, and the amount of cystine to be estimated was kept under 0.075 mg. per 25 ml. This meant that the wave produced by the cystine could not be higher than the increase of the wave height caused by the cysteine addition.

Because of the greater error of this method as compared with that using several increments of cystine, two determinations were usually made on every hydrolysate, each involving curves made with and without the addition of cystine.

As the starch in flour causes a considerable destruction of cystine during hydrolysis, it was removed by boiling the flour with a 5% solution of trichloroacetic acid. This treatment dissolved the starch and precipitated most of the protein. After isolating and washing the residue, which contained about 45% protein ( $N \times 5.7$ ), its cystine content was determined by hydrolysing for 16 hours in 20% hydrochloric acid, and making the polarographic determinations already described on the hydrolysate.

Hydrolysis was carried out with 20% hydrochloric acid (analar). Six to eight hours was found to be minimum hydrolysis time because the minimum and maximum of the current-voltage curve could not be developed in a shorter time. For convenience, a time of 16 hours (5 p.m. to 9 a.m.) was used. Under these conditions at least 90% of the cystine added before hydrolysis was always recovered.

From 100 to 200 mg. of flour protein containing 1-4 mg. cystine were hydrolyzed in 10 ml. of 20% hydrochloric acid, the humin which formed was removed by filtration and the filtrate was made up to 50 ml. with distilled water.

A current-voltage curve was made on a solution containing 10 ml. 0.2 *N* ammonium hydroxide solution, 10 ml. of a solution containing ammonium chloride (0.2 *N*) and cobalt chloride (0.002 *N*), 1 ml. of protein hydrolysate, and 4 ml. of distilled water. A current-voltage curve was also made by replacing 1 ml. of distilled water in the preceding solution with 1 ml. of a freshly prepared aqueous solution of cysteine hydrochloride (25 mg./250 ml.).

As a check on the method, a recovery experiment was conducted in which cystine was added to a pea flour protein preparation before hydrolysis. Since the cystine content of the protein of pea flour was only about one-fifth of that of wheat flour protein, the proportion of

cystine in relation to the other constituents is much more unfavorable. To 100 mg. samples of the protein precipitated from pea flour by boiling with 5% trichloroacetic acid, 0.3 and 0.6 mg. of cystine, respectively, were added and the mixture hydrolyzed by boiling with 10 ml. of 20% hydrochloric acid solution for 16 hours. After filtration the hydrolysate was brought to a pH of 5 to 6 to permit the addition of more than 1 ml. to the supporting electrolyte without appreciably changing the pH. The protein preparation contained 42.7% protein and the cystine content of the protein ( $N \times 5.7$ ) was found to be 0.76%.

In triplicate experiments the mean recoveries were 98 and 94% for the addition of 0.3 and 0.6 mg. cystine, respectively.

As a further test of the method, cystine was determined in hydrolysates of several proteins with the following results:

| Protein      | $N, \%$ | Cystine content |                       |      |
|--------------|---------|-----------------|-----------------------|------|
|              |         | Found<br>$\%$   | Value from literature |      |
|              |         |                 | $\%$                  |      |
| Fibrin       | 16.7    | 1.0             | 1.1                   | (2)  |
| Gliadin      | 15.4    | 1.6             | 2.1                   | (3)  |
| Globin (man) | 14.6    | 1.2             | 1.15                  | (15) |
| Casein       | 12.6    | 0.41            | 0.37                  | (4)  |

The values compare favorably with those given in the literature.

**Extensograms.** Extensograms representing each flour were made by mixing the flour with 2% sodium chloride and sufficient water in the Brabender Farinograph to give a dough consistency of 500 Brabender units at the point of maximum dough development. The doughs were rounded and formed into cylindrical pieces in the Brabender Extensograph as described by Munz and Brabender (11). The extensograms were made after a rest period of 135 minutes at 30°C.

The surface areas ( $\text{cm}^2$ ) of the extensograms were measured and the ratio: resistance to extensibility,  $F$  (in extensograph units)/extensibility,  $E$  (mm.), calculated. This ratio gives an impression of the shape of the extensogram (11).

## Results and Discussion

The results of the cystine determinations and extensograms for the wheat flours are summarized in Table I. The percentage cystine in wheat flour proteins reported in the literature (5) range between 1.8 to 2.1%<sup>1</sup> so that the values of 2.2 to 4.5% found in the present study

<sup>1</sup> Calculated for protein =  $N \times 5.7$

are considerably higher. This may be due to compounds of the thiazolidine carboxylic acid type being estimated as free cystine by the polarographic method. These compounds could result from the condensation of cystine with carbohydrate degradation products (12). However, when using a modified Folin-method (phospho-18-tungstic acid reagent) Gubler and Greaves (9) found 0.34–0.41% cystine in the

TABLE I  
THE RELATION BETWEEN THE PROTEIN AND CYSTINE CONTENTS OF WHEAT FLOURS AND DOUGH PROPERTIES, AS MEASURED BY THE BRABENDER EXTENSOGRAPH

| Sample<br>no.  | Protein<br>(N $\times$ 5.7)<br>% | Cystine content |            | Extensogram characteristics <sup>1</sup> |     |
|----------------|----------------------------------|-----------------|------------|------------------------------------------|-----|
|                |                                  | Protein<br>%    | Flour<br>% | Area<br>Cm.                              | F/E |
| AFRICAN FLOURS |                                  |                 |            |                                          |     |
| 1              | 11.7                             | 4.0             | 0.47       | 148                                      | 6.4 |
| 2              | 11.2                             | 4.1             | 0.46       | 100                                      | 5.8 |
| 3              | 11.1                             | 3.8             | 0.43       | 102                                      | —   |
| 4              | 11.1                             | 4.1             | 0.45       | 135                                      | 3.1 |
| 5              | 10.5                             | 4.5             | 0.47       | 129                                      | 7.5 |
| 6              | 10.4                             | 4.2             | 0.44       | 81                                       | 3.8 |
| 7              | 8.1                              | 3.5             | 0.28       | 19                                       | 1.1 |
| 8              | 7.3                              | 3.5             | 0.26       | 49                                       | 5.5 |
| 9              | 7.2                              | 3.0             | 0.22       | 31                                       | 3.2 |
| 10             | 7.1                              | 3.6             | 0.26       | 30                                       | 2.3 |
| DUTCH FLOURS   |                                  |                 |            |                                          |     |
| 11             | 9.6                              | 2.2             | 0.21       | 28                                       | 0.8 |
| 12             | 9.4                              | 4.1             | 0.38       | 68                                       | 2.7 |
| 13             | 9.1                              | 2.5             | 0.23       | 27                                       | 1.2 |
| 14             | 9.1                              | 3.9             | 0.34       | 70                                       | 4.2 |
| 15             | 8.9                              | 3.2             | 0.29       | 14                                       | 0.4 |
| 16             | 8.8                              | 2.9             | 0.26       | 29                                       | 1.0 |
| 17             | 8.8                              | 3.0             | 0.26       | 21                                       | 0.5 |
| 18             | 8.7                              | 4.5             | 0.39       | 68                                       | 4.7 |
| 19             | 8.7                              | 2.8             | 0.24       | 29                                       | 1.6 |
| 20             | 8.7                              | 3.0             | 0.26       | 20                                       | 0.6 |
| 21             | 8.4                              | 3.0             | 0.25       | 31                                       | 2.0 |
| 22             | 8.3                              | 3.0             | 0.25       | 19                                       | 0.4 |
| 23             | 8.3                              | 3.0             | 0.25       | 20                                       | 0.5 |

<sup>1</sup> The extensograms were made after a rest period of 135 minutes. F represents the ratio of the resistance to extension in Brabender units and E the extensibility in Cm.

grain. This indicates about 3.5% for flour protein, which gives better agreement with the present results.

The flour samples in Table I are arranged in order of protein content and it is readily seen, as is well known, that the area of the extensogram decreases with a decrease in protein content. The correlation coefficient between the data for these variables is +0.82. Similarly, the cor-

relation between the surface area of the extensogram and the cystine percentage of the protein is  $+0.77$ .

These results indicate that the extensogram area is a function of both the quantity of protein present and of the cystine content of the protein. If these variables are combined to give the cystine contents of the flour, an even better correlation with the surface area of the extensogram is obtained; namely,  $r = +0.92$ .

It may be concluded that the quality of flour protein for bread-making purposes increases with an increase in the number of possible  $-S-S-$  linkages, as determined by its cystine content. The total cystine, that is the total number of possible  $-S-S-$  linkages, largely determines those properties of flour which are measured by the extensogram.

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## A METHOD FOR THE DETERMINATION OF LACTOSE IN BREAD<sup>1</sup>

R. P. CHOI, C. W. TATTER, AND C. M. O'MALLEY

### ABSTRACT

A simple fermentation method has been developed for the determination of lactose and indirectly nonfat milk solids in bread. The procedure involves extracting the sugars from bread with dilute alcohol, destroying the non-lactose reducing sugars by fermenting the alcohol-free extract for 2½ hours at 30°C. with baker's yeast, and analyzing for lactose by means of the Somogyi micro-method for reducing sugars. Different brands of yeast cake and dry yeast can be used. Excellent recoveries have been obtained when varying quantities of nonfat dry milk solids were added to water bread. Likewise, results determined by two different laboratories on samples of bread of varying concentrations of lactose or nonfat dry milk solids showed good agreement between the two laboratories and also with the theoretical values.

Methods for the estimation of nonfat dry milk solids in bread are generally based upon the determination of lactose by selective fermentation with baker's yeast. Among the procedures utilizing this principle are those of Snethlage (7), Meyer (5), Schut and DeJong (6), and Magraw and Copeland (4). The last procedure was developed in this laboratory and has been adopted in *Cereal Laboratory Methods* (2). While it gives satisfactory results, it is time consuming and somewhat objectionable because it requires the use of hydrogen sulfide.

A shorter and less tedious method is presented in this report. Extraction of the lactose is accomplished with dilute ethanol, a procedure which eliminates the starches and nearly all the proteins. Consequently, it is not necessary to use alpha-amylase or repeated protein precipitations in subsequent operations. Also, by using a small aliquot along with continuous agitation, the time required for complete fermentation of non-lactose reducing sugars is reduced to two and one-half hours. Finally, substitution of the micro-method of Somogyi for the Munson-Walker method for lactose determination further decreases the time required per analysis.

### Materials and Methods

**Reagents.** 1. Yeast suspension. This was prepared according to the procedure of Jones (3). Commercial baker's yeast was washed by

<sup>1</sup> Manuscript received February 24, 1950.

Contribution from American Dry Milk Institute, Inc., Chicago, 1, Illinois.

centrifuging with three to four times its volume of distilled water until the supernatant liquid was clear. The yeast was finally diluted to a 25% suspension and stored in a refrigerator at 0° to 4°C. for 24 hours before use. Preparations as old as one week could still be used.

2. Yeast nutrient solution. 1.7 g. bacto-peptone (Difco Laboratories), 0.50 g. dipotassium phosphate, and 0.33 g. magnesium sulfate heptahydrate were dissolved in water and diluted to 100 ml.

3. Protein precipitant. 50 g. sodium tungstate and 6 g. disodium phosphate were dissolved in 200 ml. distilled water. After adding slowly 220 ml. of 2 *N* hydrochloric acid, the solution was mixed and diluted to 500 ml.

4. Somogyi's reagent (8). 12 g. Rochelle salt, 20 g. anhydrous sodium carbonate, and 25 g. sodium bicarbonate were dissolved in about 500 ml. distilled water. To this was added with stirring 6.5 g. copper sulfate pentahydrate previously dissolved in 100 ml. water. In a separate container, 10 g. potassium iodide, 0.800 g. potassium iodate, and 18 g. potassium oxalate were dissolved in about 200 ml. water. The two solutions were mixed and diluted to 1 l.

5. Sodium thiosulfate, 0.005 *N*. Since this solution cannot be kept unchanged for long, it was prepared every day by diluting freshly standardized 0.1 *N* solution.

6. Sodium hydroxide, 0.5 *N*.

7. Sulfuric acid, 2 *N*.

8. Starch solution, 1%.

*Procedure.* The bread to be analyzed was sliced, air-dried, and then crushed to fine particles. The moisture content of the air-dried sample was determined by the A.O.A.C. vacuum oven method (1). A sample of 15 g. of the air-dried bread was weighed into a 200 ml. volumetric flask containing 60 ml. water. After thorough mixing, 35 ml. 95% ethanol were added and the sample immersed in a boiling water bath for 15 minutes. When cooled to room temperature, the content was diluted to volume with 95% ethanol, transferred to a centrifuge bottle, and centrifuged for 10 minutes at approximately 1,000 r.p.m. One hundred and fifty milliliters of the supernatant liquid were evaporated to about 40 ml. and then diluted to 100 ml. in a volumetric flask.

For fermentation, 10 ml. of this bread extract were transferred to a 50 ml. Erlenmeyer flask; 6 ml. yeast suspension and 5 ml. yeast nutrient solution were added. A blank test was made using 10 ml. of water in place of the bread extract. The flask was stoppered with a one-hole rubber stopper fitted with a piece of 6 mm. glass tubing about 10 cm. long and shaken at a moderate rate for 2.5 hr. in a constant temperature water bath at 30°C. The fermented sample was then

transferred to a 50 ml. centrifuge tube and centrifuged for 10 minutes at approximately 1,000 r.p.m. The supernatant layer was decanted into a 50 ml. volumetric flask and the residue washed twice with 10 ml. portions of water. The washings were combined with the decanted liquid. To this solution was next added, with shaking, 2.5 ml. protein precipitant. After diluting to volume and mixing, the mixture was filtered, discarding the first few milliliters of the filtrate.

For lactose determination, 5.00 ml. of the clear filtrate were pipetted into a Pyrex culture tube (22 × 175 mm) and neutralized to the phenol red end-point with 0.5 *N* sodium hydroxide. To this, 5.00 ml. Somogyi's reagent and two drops of benzene were added. The tube was capped with a glass bulb and immersed in a vigorously boiling water bath for exactly 15 minutes after which the contents were cooled without agitation in a cold water bath. Then, 2.5 ml. 2 *N* sulfuric acid were added. When the cuprous oxide precipitate was completely dissolved, the excess iodine was titrated with 0.005 *N* sodium thiosulfate using starch as an indicator. The difference between the titration value of the blank and that of the sample was referred to a standard curve to determine the quantity of lactose present. The reference curve was established by testing 5.00 ml. portions of lactose solutions containing from 0 to 5 mg. of lactose hydrate with 5.00 ml. portions of Somogyi's reagent and plotting the difference between the titration value of the blank and that of the lactose solution against the corresponding lactose content.

From the amount of lactose found in the 5.00 ml. aliquot of the fermented sample, the percentage of lactose present in the moisture-free bread can be calculated as follows:

$$\% \text{ lactose} = \frac{187}{150} \times \frac{100}{10} \times \frac{50}{5} \times \frac{100 \times L}{15(100 - M)} \times 100 = \frac{8.33 \times L \times 10^4}{100 - M}$$

"L" is the grams of lactose in the 5.00 ml. aliquot and "M" is the per cent moisture in the air-dried bread. The factor 187 is the total volume of dilute alcohol in 200 ml. of bread suspension, using 13 ml. as the volume occupied by 15 g. of air-dried bread, a factor employed by Magraw and Copeland (4). A blank of 0.35% lactose was subtracted from the final result. This is a correction for the amount of non-fermentable reducing material present in bread containing no milk and is about the same as that found by the above workers (4). To convert to nonfat dry milk solids, which is the usual source of lactose in bread, the per cent of lactose is multiplied by two, since the average lactose content of nonfat dry milk solids is approximately 50%. A sample of dry bread of known lactose content may be run simultaneously with unknown breads, if desired, to check the reliability of each series of analyses.

### Results and Discussion

Except where otherwise noted, samples of bread for this study were prepared by our baking laboratory, using the following formula. The straight-dough technique was followed:

|       |        |                                  |         |
|-------|--------|----------------------------------|---------|
| Flour | 300 g. | Yeast food                       | 0.75 g. |
| Water | 210 g. | Malt                             | 1.5 g.  |
| Sugar | 12 g.  | Shortening                       | 6 g.    |
| Salt  | 6 g.   | Nonfat dry milk solids, variable |         |
| Yeast | 6 g.   |                                  |         |

In accordance with general baking practice, the percentage of nonfat dry milk solids or lactose added is expressed as per cent of flour used.

Using an extract prepared from a bread containing 6% nonfat dry milk solids, the time necessary for complete fermentation of non-lactose reducing sugars was determined by fermenting 10 ml. portions for varying periods at 30°C. with shaking. From the data plotted in

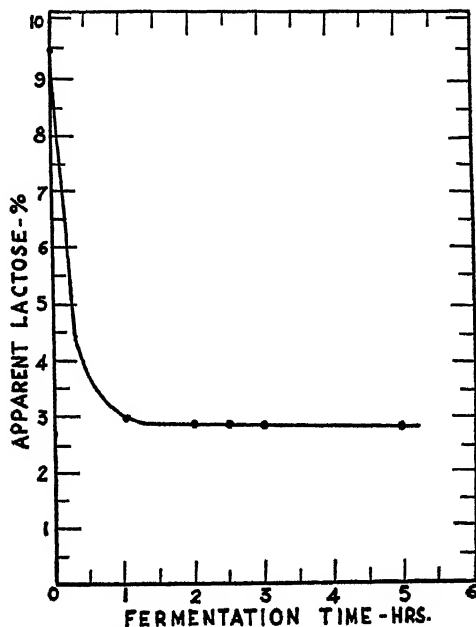


Fig. 1. Fermentation time necessary for the complete destruction of non-lactose reducing substances on extract of bread containing 6% of nonfat dry milk solids (2.89% lactose).

Fig. 1 it can be noted that fermentation is practically complete after 1 hour. A 2½ hour period was chosen to insure complete fermentation. In the absence of agitation, a much slower rate of fermentation resulted.

Recovery experiments were made by adding to an air-dried water-bread, nonfat dry milk solids (51.2% lactose) in amounts to give 0,



TABLE I  
RECOVERY OF NONFAT DRY MILK SOLIDS<sup>1</sup> ADDED TO WATER BREAD

| Nonfat dry milk solids added | Lactose, theoretical | Lactose found |         |         |
|------------------------------|----------------------|---------------|---------|---------|
|                              |                      | Trial 1       | Trial 2 | Trial 3 |
| %                            | %                    | %             | %       | %       |
| 0                            | 0                    | 0             | 0.11    | 0.17    |
| 2                            | 1.02                 | 1.07          | 1.12    | 1.11    |
| 4                            | 2.05                 | 2.13          | 2.20    | 2.20    |
| 6                            | 3.08                 | 3.21          | 3.21    | 3.19    |
| 8                            | 4.10                 | 4.09          | 4.09    | 3.91    |
| 12                           | 6.15                 | 5.92          | 6.13    | 6.13    |

<sup>1</sup> Containing 51.2% lactose by analysis.

2, 4, 6, 8, and 12% nonfat milk solids. These mixtures were analyzed for lactose by the present method. Results of three separate trials are presented in Table I. Good agreement is obtained between the experimental and the theoretical values. The average deviation of all results from their corresponding theoretical results is 0.092% lactose, calculated without regard to sign.

A series of breads containing varying quantities of lactose or nonfat dry milk solids was prepared by the American Institute of Baking by the sponge dough technique following a formula similar to that given above. These breads were analyzed for lactose at the American Institute of Baking and in our own laboratory, using the present method. From the total weight of the bread after air drying and from the moisture content, the theoretical lactose value on the moisture-free basis was calculated. Results are presented in Table II.

TABLE II  
COLLABORATIVE RESULTS ON BREADS CONTAINING VARYING AMOUNTS OF LACTOSE OR NONFAT DRY MILK SOLIDS

| Lactose content <sup>1</sup> | Lactose content (moisture-free basis) |        |            |
|------------------------------|---------------------------------------|--------|------------|
|                              | Theoretical                           | A.I.B. | ADMI       |
| %                            | %                                     | %      | %          |
| 0                            | 0                                     | —      | 0.0        |
| 0                            | 0                                     | 0.12   | 0.01       |
| 2                            | 1.96                                  | —      | 1.75       |
| 4                            | 3.82                                  | 3.67   | 3.60       |
| 6                            | 5.96                                  | 5.84   | 5.48, 5.42 |
| 1                            | 0.97                                  | 0.80   | 0.86       |
| 2                            | 1.91                                  | 1.56   | 1.74       |
| 3                            | 2.89                                  | 2.71   | 2.84, 2.86 |
| 4                            | 3.98                                  | 3.75   | 3.80       |

<sup>1</sup> In the first five samples listed lactose was added; in the last four nonfat dry milk solids was used.

The agreement between results from the two laboratories, as well as between the observed and the theoretical results, is satisfactory.

In most of these experiments Fleischmann baker's yeast (one-pound size) was used. However, when other brands of yeast, both in the cake form and in the dry form, were tried, they were found to be equally satisfactory as indicated by the data in Table III for two

TABLE III  
RESULTS WITH DIFFERENT BRANDS OF YEAST

| Yeast                                 | Lactose found, % (moisture-free basis) |                            |
|---------------------------------------|----------------------------------------|----------------------------|
|                                       | 4% Lactose bread                       | 8% ndms <sup>1</sup> bread |
| Fleischmann (pound cake)              | 4.14                                   | 3.98                       |
| Fleischmann ( $\frac{3}{4}$ oz. cake) | 3.72                                   | 4.15                       |
| Fleischmann (1 oz. cake)              | 4.00                                   | 4.20                       |
| Fleischmann (dry)                     | 4.16                                   | 4.15                       |
| Red Star ( $\frac{3}{4}$ oz. cake)    | 4.11                                   | 4.13                       |
| Red Star (dry)                        | 3.95                                   | 3.95                       |
| National (2 oz. cake)                 | 4.16                                   | 3.89                       |
| National (dry)                        | 4.16                                   | 3.97                       |

<sup>1</sup> Nonfat dry milk solids

breads analyzed on different occasions with different samples of yeasts. In using the dry yeasts, sufficient water was added to give a suspension of approximately the same concentration as the 25% suspension prepared from yeast cakes.

The precision of the present method was estimated by analyzing a white bread containing 6% nonfat milk solids eight separate times. The standard deviation was found to be 0.12% lactose, which, assuming normal distribution, would mean that approximately 95% of all determinations should fall within  $\pm 0.24\%$  lactose from the mean value. An indication of the accuracy of the method can be obtained from the recovery results shown in Tables I and II. With the exception of two results, all results are within  $\pm 0.24\%$  lactose of the theoretical values. In comparison, the accuracy of the Magraw-Copeland procedure has been given as  $\pm 0.5\%$  nonfat dry milk solids or  $\pm 0.25\%$  lactose.

#### Acknowledgment

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## INHIBITION OF BETA-AMYLASE BY CYANIDE<sup>1</sup>

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### ABSTRACT

Addition of sodium cyanide to malt extract solutions markedly inhibited the saccharification produced by these extracts. The cyanide addition had no effect on alpha-amylase activities but lowered apparent beta-amylase activities of these extracts. Sodium cyanide completely inhibited the saccharifying activities of extracts from ungerminated soybeans, wheat and barley, and of solutions of a commercial beta-amylase. Cyanide, therefore, has a differential effect upon amylase complexes, inhibiting the beta-amylase. This inhibition is complete when beta-amylase is the only amylolytic enzyme present, but apparently not with extracts of malt.

Inhibition or destruction of enzyme activity by chemical reagents is frequently encountered. An inhibiting agent may sometimes be employed to advantage in the study of enzyme systems for the inhibition of the activity of certain enzyme components without affecting others.

In the course of studies on the enzyme systems of bacteria and molds, interest arose in finding substances which would have a differential inhibiting effect upon components of the amylase complex. Although a number of reports on amylase inhibitors have appeared, in most cases these have been unidentified substances of natural origin (1, 5, 7, 14). However, Purr (12) found that ascorbic acid inhibited beta-amylases but did not affect the alpha-amylases of plants. Hanes (2), Weidenhagen and Lu (15), and Janicki (3) likewise found that ascorbic acid inhibited the action of beta-amylase, and Hanes reported that reductone and dihydroxymaleic acid also inhibited beta-amylase.

Cyanides have frequently been employed for inhibiting various enzymes, particularly in metabolic studies. There are few reports on

<sup>1</sup> Manuscript received February 20, 1950. Contribution from the Chemistry Department and Industrial Science Research Institute, Iowa State College, Ames, Iowa.

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the effect of cyanide upon amylases, especially upon the effect of cyanide upon the individual components of amylase complexes. Myrbäck (8) found that potassium cyanide in small amounts strongly inhibited the activity of salivary amylase, whereas equimolar concentrations of hydrogen cyanide caused much less inhibition. However, 0.1 *N* hydrogen cyanide almost completely destroyed the activity of the enzyme. Ninomiya (10) found that salivary amylase was somewhat inhibited by potassium cyanide or potassium thiocyanate, even in concentrations as low as 0.0005 *M*. Nguyen-Van-Thoi and Silhol-Bernere (9) reported that purified pancreatic amylase treated at pH 6.8 at 40°C. for one-half hour with 0.001 to 0.1 *M* potassium cyanide was not affected, whereas the crude enzyme was inhibited by this reagent at very low concentrations.

Since salivary amylase and pancreatic amylases are predominately of the alpha-amylase type, tests were made on the inhibiting action of cyanide upon certain enzyme extracts having alpha-amylase activity. Preliminary tests showed that cyanide markedly inhibited the amylolytic activity of malt extracts but had relatively little effect upon fungal amylase solutions. Further work, reported in this paper, showed that cyanide inhibits beta-amylase without affecting activities of other components of the amylase complex.

### Materials and Methods

The amylolytic materials employed in this investigation included samples of malts, grains, and a commercial beta-amylase preparation. Three malts, kindly supplied by Dr. Eric Kneen of the Kurth Malting Company, of widely varying alpha-amylase and beta-amylase activities were used. One of these, malt A, was a "high-kilned malt," low in beta-amylase and limit dextrinase activities. Two varieties of soybeans and one sample each of wheat and barley were supplied by the Agronomy Department of Iowa State College. The commercial beta-amylase preparation was "beta-amylase for analytical purposes" purchased from Wallerstein Laboratories.

Extracts were prepared from the malts and grains for testing in the following manner: The materials were finely ground in a burr mill and 5 g. of each extracted with 100 ml. of water for 1 hour at 30° C., with frequent shaking. The extracts were then centrifuged and filtered. Where cyanide treatment was employed, 50 mg. of sodium cyanide was added to 50 ml. of extract, which corresponds to 0.1% or 0.02 *M* concentration of the cyanide, and the solution held for 1 hour at 30° C.

Alpha-amylase determinations were made by the method of Sandstedt, Kneen, and Blish (13) using the cobalt-chromate color

standard of Olson, Evans, and Dickson (11). Results of the analyses were calculated in terms of the alpha-dextrinogenic units of the former authors, representing the number of grams of soluble starch which, under the influence of an excess of beta-amylase, are dextrinized by one gram of malt in one hour at 30°C.

Overall saccharification values and beta-amylase activities were determined by the method of Kneen and Sandstedt (4). The results were calculated in terms of saccharogenic units, representing the number of grams of soluble starch converted to maltose by one gram of material in one hour at 30°C., and beta-amylase units, representing the number of grams of soluble starch converted to maltose by the beta-amylase of one gram of material in one hour at 30°C. Independent tests showed that the amounts of cyanide employed affected the quantities of apparent maltose found by the ferricyanide method by from 0 to 2%, depending upon the sugar concentration. Such differences are not significant for the comparisons involved in this investigation.

### Results and Discussion

The procedures devised by Sandstedt, Kneen, and Blish (13) and by Kneen and Sandstedt (4) for differential estimation of alpha-amylase and beta-amylase in malt were adapted to the investigation of the inhibiting action of cyanide upon these components of malt amylase. Extracts of the three malts were prepared and portions of each were treated with sodium cyanide as described above. The cyanide-treated and untreated extracts were then analyzed. The data for the malt samples are given in Table I.

TABLE I

EFFECT UPON AMYLOLYTIC ACTIVITY OF TREATING AQUEOUS EXTRACTS OF MALTS WITH 0.1% SODIUM CYANIDE

| Malt sample | Untreated extract         |                     |                    | Cyanide-treated extract   |                     |                    |
|-------------|---------------------------|---------------------|--------------------|---------------------------|---------------------|--------------------|
|             | Alpha-dextrinogenic units | Saccharogenic units | Beta-amylase units | Alpha-dextrinogenic units | Saccharogenic units | Beta-amylase units |
| A           | 34                        | 5.34                | 3.65               | 34                        | 2.66                | 0.97               |
| B           | 64                        | 19.37               | 16.33              | 63                        | 14.32               | 11.33              |
| C           | 46                        | 9.67                | 7.42               | 45                        | 7.36                | 5.16               |

The results of this experiment indicated that cyanide had little if any effect upon the alpha-amylase activity of the malt extracts, but markedly lowered the overall saccharification produced by the extracts. However, apparent residual beta-amylase activity, as calculated from the analytical data, still existed.

The effect of cyanide treatment of aqueous extracts of soybeans, wheat, and barley was then investigated. Since extracts of these ungerminated grains are good sources of beta-amylase, while alpha-amylase is essentially absent, only saccharogenic values were determined on the cyanide-treated and untreated extracts. Simultaneously with the grain extracts, solutions of three different concentrations of the commercial beta-amylase were prepared, a portion of each treated with 0.1% sodium cyanide in the same manner as for the grain extracts, and saccharogenic values determined on the solutions. Data from these determinations are given in Table II.

TABLE II  
EFFECT UPON AMYLOLYTIC ACTIVITY OF TREATING SOLUTIONS FROM SOURCES OF  
BETA-AMYLASE WITH 0.1% SODIUM CYANIDE

| Sample                   | Saccharogenic units |                 |
|--------------------------|---------------------|-----------------|
|                          | Untreated           | Cyanide-treated |
| Soybean A                | 10.03               | 0               |
| Soybean B                | 9.00                | 0               |
| Wheat                    | 4.90                | 0               |
| Barley                   | 5.56                | 0               |
| Commercial beta-amylase: |                     |                 |
| 0.2% solution            | 2.66 <sup>1</sup>   | 0               |
| 0.5% solution            | 6.67 <sup>1</sup>   | 0               |
| 1.0% solution            | 13.55 <sup>1</sup>  | 0               |

<sup>1</sup> Units per ml. of solution

The results presented in Table II show conclusively that beta-amylase from the sources tested is completely inhibited or destroyed by sodium cyanide. Even solutions of the commercial beta-amylase in the relatively high concentration of 1.0% showed no saccharifying activity after cyanide treatment.

Referring again to the data of Table I, it may be noted that although overall saccharification was markedly lowered by treatment of the malt extracts with cyanide, apparent residual beta-amylase activity was still appreciable. This was especially true for malts B and C. Since cyanide completely destroyed the beta-amylase of the ungerminated grains and commercial beta-amylase, it would be assumed that the beta-amylase activity of the malts should also be completely inhibited. It is possible, however, that other constituents in the extracts may exert a protective action on the beta-amylase of malts. Probably other components of the malt amylase system, such as limit dextrinase, are not inactivated and together with the alpha-amylase are responsible for much of the saccharification produced. The apparent residual beta-amylase activity may possibly be due, at least in part, to limit dextrinase. The contribution which limit dex-

trinas makes to total saccharification needs further investigation to elucidate this point. However, the hypothesis is strengthened by the fact that the apparent beta-amylase activity for malt A after cyanide treatment was relatively low. Malt A was a "high-kilned malt," so treated for destruction of beta-amylase and limit dextrinase. Such treatment does not entirely eliminate these components, but markedly reduces them according to Kneen and Spoerl (6). The data show relatively low beta-amylase activity for this malt before cyanide treatment, and very low residual apparent beta-amylase activity after cyanide treatment.

It may be concluded from the work reported in this paper that cyanide has a differential effect upon amylolytic enzyme complexes, inhibiting only the activity of beta-amylase. When beta-amylase is present as the sole amylolytic component it is completely inhibited by cyanide. The apparent beta-amylase activity of malts is apparently only partially inhibited by cyanide. Further work will be necessary to fully establish the degree of inhibition of beta-amylase in malts and in other mixtures of the several amylase components.

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## EXPERIMENTS WITH AMMONIUM PERSULFATE AS A FLOUR MATURING AGENT <sup>1</sup>

J. M. DOTY AND R. C. SHERWOOD <sup>2</sup>

### ABSTRACT

Ammonium persulfate in bread flours at levels of 1 to 20 g. per 100 pounds of flour showed a definite maturing action. Loaf volumes and crumb texture scores were improved by ammonium persulfate in spring, winter, and west coast bread flours. Ammonium persulfate treated flours showed good storage properties and compared favorably in baking properties with nitrogen trichloride treated flours, both freshly milled and after several months' storage.

Ammonium persulfate additions to flour did not affect the thiamine and riboflavin content of enriched flour. No deleterious effect could be observed on the vitamin content of persulfate-treated, enriched flour after storage, either in the flour or in the bread made from the flour.

Ammonium persulfate has been used as a flour maturing agent in the production of bread for many years in Europe. Very few reports appear in the literature. Kent-Jones and Amos (1) have reported its maturing properties, and its use in England and on the Continent for many years, stating that persulfates cause improvement in elasticity and stability of dough, and their use results in superior breads. Because certain changes are being made in the use of maturing gases in the production of flour, investigations into the use of ammonium persulfate in maturing flour are herewith reported.

Ammonium persulfate is a powerful oxidizing agent, containing 7% active oxygen. It is a white crystalline compound which can be blended with relatively inert compounds and added directly to flour or flour doughs. Since the persulfate is water soluble, it goes into solution promptly when a dough is mixed and exerts its oxidizing action without delay.

This report shows the use of ammonium persulfate in maturing bakers flour made from hard red winter wheat, spring wheat, and west coast hard wheats.

### Materials and Methods

Baking results are reported on a modified A.A.C.C. procedure using 100 g. of flour per loaf. The ingredients used in the modified formula include yeast, salt, sugar, shortening, milk, malt, and flour. All results

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<sup>2</sup> Sterwin Chemicals Inc. Work done in Doty Laboratories, Kansas City, Missouri.



obtained on 100 g. flour loaves were verified by the sponge-dough procedure producing regular 1-pound commercial-type loaves.

Over 50 southwestern winter wheat bakers flours have been treated with ammonium persulfate and compared with flours given regular commercial nitrogen trichloride maturing. Many spring wheat bakers flours and west coast bakers flours have also been compared in this manner. For this report, a typical example has been chosen from each of the three groups of bakers flours tested.

Ammonium persulfate was added to flour both in the dry form and in solution at the dough mixing stage. The dry form was a fine-ground mixture of 50% ammonium persulfate and 50% inert carrier such as calcium sulfate.

All flour used in storage tests was stored in cloth bags on open shelves at room temperature in the laboratory. Sacks were opened for analytical work, re-tied, and returned to shelf for further storage.

### Results and Discussion

Southwest bakers flour untreated and with various increments of nitrogen trichloride or ammonium persulfate are reported in Table I. The optimum nitrogen trichloride treatment for this flour is in the neighborhood of 1 g. per 100 pounds of flour, which compares closely with 4 g. of ammonium persulfate per 100 pounds of flour. Loaf volumes, texture scores, and over-all bread scores show similar results

TABLE I  
BAKING RESULTS ON HARD WINTER WHEAT BAKERS FLOUR COMPARING  
AMMONIUM PERSULFATE AND NITROGEN TRICHLORIDE  
AS MATURING AGENTS

| Treatment per 100 lbs. flour         | Loaf volume | Texture score | Total bread score |
|--------------------------------------|-------------|---------------|-------------------|
| Unmatured                            | 635         | 50            | 59                |
| $\frac{1}{2}$ gram N.T. <sup>1</sup> | 735         | 70            | 78                |
| 1 gram N.T.                          | 840         | 90            | 95                |
| 2 grams N.T.                         | 800         | 70            | 73                |
| 2 grams A.P. <sup>2</sup>            | 750         | 80            | 86                |
| 4 grams A.P.                         | 850         | 89            | 95                |
| 8 grams A.P.                         | 810         | 75            | 78                |

<sup>1</sup> N.T.—Nitrogen Trichloride

<sup>2</sup> A.P.—Ammonium Persulfate.

between the maturing action of nitrogen trichloride and ammonium persulfate. These data are typical of results obtained on over 50 samples of southwestern winter wheat flours.

The effect of various increments of nitrogen trichloride and ammonium persulfate on untreated spring wheat bakers flour is reported in Table II. The optimum nitrogen trichloride treatment is about 1.0 g.

TABLE II  
BAKING RESULTS ON SPRING WHEAT BAKERS FLOUR COMPARING  
AMMONIUM PERSULFATE AND NITROGEN TRICHLORIDE  
AS MATURING AGENTS

| Treatment per 100 lbs flour | Loaf volume | Texture score | Total bread score |
|-----------------------------|-------------|---------------|-------------------|
|                             | cc          |               |                   |
| Unmatured                   | 700         | 65            | 71                |
| ½ gram N.T. <sup>1</sup>    | 875         | 90            | 91                |
| 1 gram N.T.                 | 880         | 97            | 97                |
| 1½ grams N.T.               | 800         | 73            | 77                |
| 2 grams A.P. <sup>2</sup>   | 850         | 81            | 85                |
| 3 grams A.P.                | 870         | 92            | 94                |
| 4 grams A.P.                | 860         | 91            | 92                |

<sup>1</sup> N.T.—Nitrogen Trichloride.

<sup>2</sup> A.P.—Ammonium Persulfate.

per 100 pounds of flour and the optimum treatment with ammonium persulfate, somewhere near 3 g. per 100 pounds of flour. Here again loaf volumes, texture scores, and over-all bread scores are similar for the optimum treatments with nitrogen trichloride or ammonium persulfate. These data are typical of many spring wheat flours tested in like manner.

Pacific coast bakers flour with various increments of nitrogen trichloride or ammonium persulfate is reported in Table III. Results are similar to those obtained on southwest bakers flour.

From these baking results, it is apparent that ammonium persulfate has definite value in artificially aging bread flours. In order to determine whether ammonium persulfate has practical application, storage tests were conducted. Finely-ground ammonium persulfate was mixed with an equal quantity of a mixture of calcium sulfate and calcium phosphate in order to make a free-flowing material suitable for mixing in flour. The flour used for the storage tests required 6 g. of ammonium

TABLE III  
BAKING RESULTS ON WEST COAST BAKERS FLOUR COMPARING  
AMMONIUM PERSULFATE AND NITROGEN TRICHLORIDE  
AS MATURING AGENTS

| Treatment per 100 lbs. flour | Loaf volume | Texture score | Total bread score |
|------------------------------|-------------|---------------|-------------------|
|                              | cc.         |               |                   |
| Unmatured                    | 610         | 50            | 57                |
| ½ gram N.T. <sup>1</sup>     | 715         | 69            | 75                |
| 1 gram N.T.                  | 755         | 88            | 89                |
| 2 grams N.T.                 | 705         | 75            | 80                |
| 2 grams A. P. <sup>2</sup>   | 700         | 70            | 75                |
| 4 grams A.P.                 | 760         | 89            | 88                |
| 8 grams A.P.                 | 710         | 77            | 80                |

<sup>1</sup> N.T.—Nitrogen Trichloride.

<sup>2</sup> A.P.—Ammonium Persulfate.

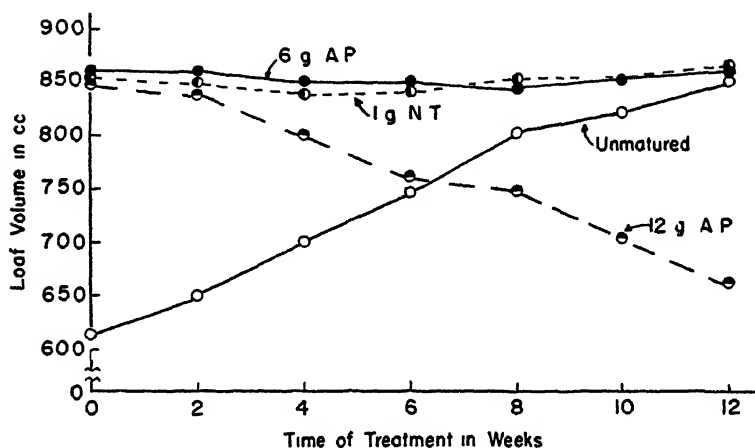


FIG. 1. Loaf volumes of flours during storage

persulfate per 100 pounds of flour to produce optimum baking results. Using the 50% mixture, the flour was treated at two different levels, one representing 6 g., and the other 12 g. of ammonium persulfate per 100 pounds of flour. Twice optimum treatment was used deliberately in the second flour to determine the degree of damage resulting from over-maturing with ammonium persulfate when fresh and after storage.

The baking results obtained with these two flours compared with the same flour untreated, and treated with nitrogen trichloride, are reported in Fig. 1. The 6 g. ammonium persulfate treated flour and

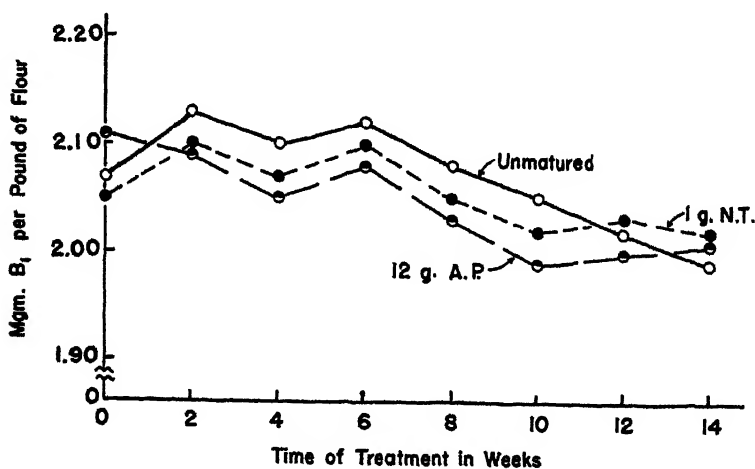


FIG. 2. Thiamine content of flours during storage.

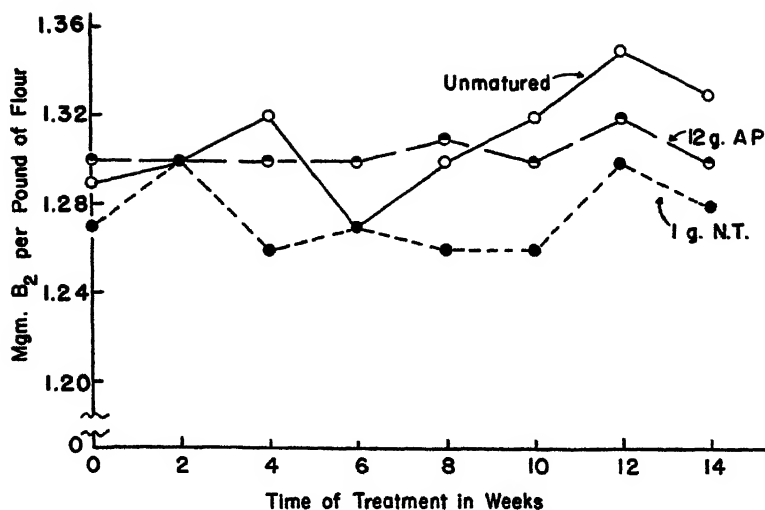


FIG. 3. Riboflavin content of flours during storage.

the nitrogen trichloride treated flour paralleled very closely over three months' storage period. As would be expected, the over-treatment with persulfate showed a decrease in loaf volume and the untreated flour showed an increase in loaf volume over the three months' period.

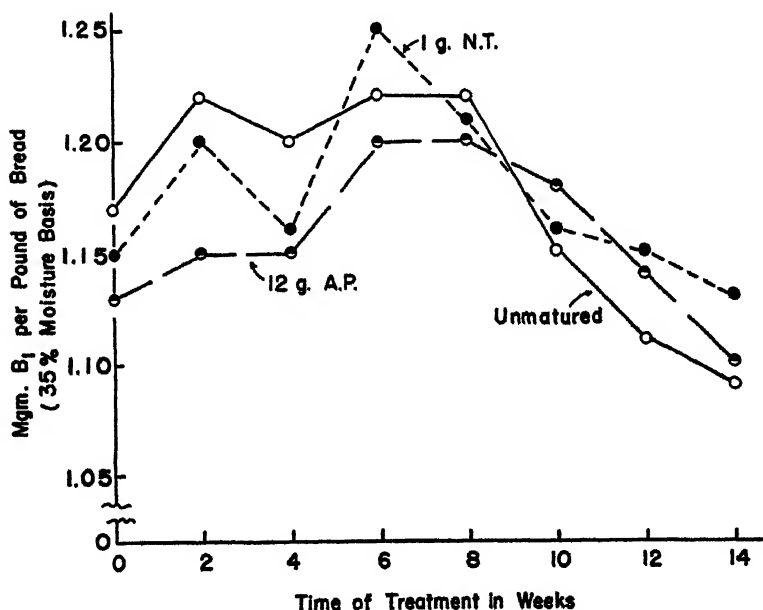


FIG. 4. Thiamine content of bread made with stored flour.

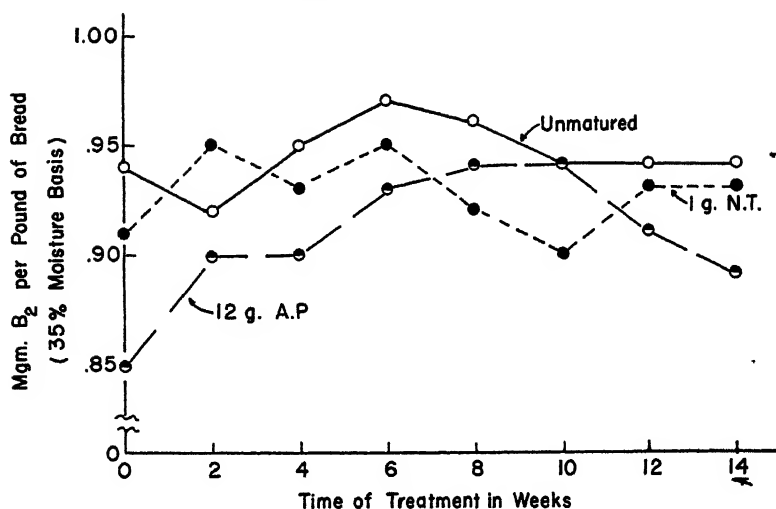


FIG. 5. Riboflavin content of bread made with stored flour.

The optimum ammonium persulfate treated flour produced satisfactory baking results over several months' storage period.

To determine the effect of ammonium persulfate on the vitamins of enriched flour, the flours of Fig. 1 were also assayed over the storage period for thiamine and riboflavin content. In Fig. 2, the thiamine content of the unenriched, the 12 g. ammonium persulfate treated, and the 1 g. nitrogen trichloride treated flours are shown. All results in this figure are well within experimental error. In Fig. 3, the riboflavin content assayed over three months' storage period is reported on the same three flours and is within experimental error.

Unenriched ammonium persulfate treated flour, nitrogen trichloride treated flour, and unenriched flour were baked using enrichment tablets in the doughs and the resulting bread assayed. The bread was air dried and assayed at a low moisture level but calculated to a 35% moisture basis. The dried bread crumbs were stored in slip-covered tin containers during the entire series of tests. The thiamine assay values are reported in Fig. 4 and the riboflavin values in Fig. 5. From these results, it is concluded there is no appreciable effect of the maturing materials upon thiamine and riboflavin content.

#### Acknowledgments

Acknowledgment is given to D. K. Dubois, C. A. Brockman, and H. L. Marks, who assisted in the experimental work reported.

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## COMPOSITION OF THE COMPONENT PARTS OF THE SORGHUM KERNEL<sup>1,2</sup>

J. E. HUBBARD, H. H. HALL, AND F. R. EARLE

### ABSTRACT

Grain from five varieties of sorghum was separated into endosperm, germ, and bran, and the proportion of each was determined. The kernel contains about 82% endosperm, 10% germ, and 8% bran. All fractions and the whole grain of each variety were analyzed for ash, protein, oil, and starch with the following average results (moisture-free basis): Whole grain 1.65, 12.3, 3.6, 73.8%; endosperm 0.37, 12.3, 0.6, 82.5%; germ 10.36, 18.9, 28.1, 13.4%; bran 2.02, 6.7, 4.9, 34.6%, respectively. The assays for niacin, panthothenic acid, riboflavin, biotin, and pyridoxine gave for the whole grain 45.3, 10.4, 1.3, 0.20, 4.7  $\mu\text{g./g.}$ ; endosperm 43.7, 8.7, 0.9, 0.11, 4.0  $\mu\text{g./g.}$ ; germ 80.7, 32.2, 3.9, 0.57, 7.2  $\mu\text{g./g.}$ ; and bran 44.0, 10.0, 4.0, 0.35, 4.4  $\mu\text{g./g.}$ , respectively. The whole grain was analyzed for crude wax by a new method and was found to contain 0.32%.

Grain sorghums have been grown extensively for many years in areas of the Southwest where climatic conditions make corn production uncertain. Such adaptation of sorghum makes it important as forage and feed in Texas, Kansas, Oklahoma, California, New Mexico, Nebraska, and Colorado. Development of dwarf types suitable for machine harvesting and of new varieties adapted to wider areas has steadily increased production until in 1949 it exceeded 152 million bushels.

While still primarily a feed crop, the large amounts of grain sorghums processed during the war by the distilling and milling industries demonstrated the value of the crop as an industrial material. Information concerning the production, composition, and use of sorghums has been compiled by Edwards and Curtis (7) and Hightower (8). The B-complex vitamin content of a number of varieties has been reported by Tanner, Pfeiffer, and Curtis (14). Some data on the composition of kafir, milo, and feterita and their component parts have been reported by Bidwell (3) and Bidwell, Bopst, and Bowling (4).

The composition of the component parts of the sorghum kernel is of importance to industries utilizing the crop because it determines the value of derivatives and by-products for feed and food uses. To supply

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Report of a study in which certain phases were carried on under the Research and Marketing Act of 1946.

<sup>2</sup> Contribution from Northern Regional Research Laboratory, Peoria, Illinois.

One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

further information on this, separation and analyses were made on grain of several varieties of sorghums.

Determinations were made of the amount of endosperm, germ, and bran, and these fractions were analyzed for ash, protein, oil, and starch, and five of the B-complex vitamins. Varieties selected for analysis are in commercial production and include Cody (waxy kafir), Pink Kafir, and the three yellow milos—Westland, Midland, and Martin. Four of the varieties selected are representative of the sorghum producing area of Kansas—Westland being a composite of samples from 11 counties, Midland a composite from 12 counties, Cody a composite from 11 counties, and Pink Kafir from 3 counties. Martin was from a large lot of Texas certified seed grown near San Antonio. All samples were grown in 1945.

### Methods

As a check on the uniformity of the samples and the accuracy of the separations, each sample was divided into four portions of approximately 20 g. each. Each portion was inspected and picked to include only sound whole kernels. Distilled water at room temperature was used to soak the grain sufficiently to facilitate removal of the bran and separation of the germ from the endosperm. Three to seven minutes were usually sufficient when the initial moisture content of the grain was 12 to 14%. Fractions were separated by hand with a scalpel, checked visually for completeness of separation, and air dried before weighing. The replications for physical proportions agreed within each variety within about 1% and were combined for chemical analysis. It was estimated initially that 5 g. of bran, the smallest fraction, would provide sufficient material for the determination of oil, protein, and vitamins, and, accordingly, only enough grain was separated to provide that quantity.

Whole grain and endosperm fractions were ground for analysis in a special laboratory hammer mill (10) with a 1/16-inch perforated screen. Bran fractions were ground in a micro Wiley mill with a 20-mesh screen. Germ was analyzed without preliminary grinding.

The grain and the separated fractions were analyzed for moisture, ash, nitrogen, oil, and starch. Methods of the Association of Official Agricultural Chemists (1) were used to determine moisture, ash, and nitrogen. Oil was determined by the A.O.A.C. method for oil in cottonseed, except that the samples of germ were extracted about six hours, ground in a mortar, and then re-extracted overnight. Starch was determined by the Clendenning polarimetric method (5) with uranyl acetate being used in place of stannic chloride as the protein precipitant.

Crude wax was determined by a simple procedure by use of hot benzene as solvent. Thirty to 60 g. samples of whole grain were placed

on small squares of cheesecloth and the corners of the cloth gathered to make a bag. The sample was dipped for ten seconds into 100 ml. of benzene maintained near the boiling point on a steam bath, drained momentarily, and dipped twice more. The series of three immersions was repeated with a fresh portion of hot benzene. After filtration through fritted glass funnels, the solutions were evaporated to dryness, cooled, and weighed. About 85 to 90% of the easily extractable material was recovered in the first solution and about 10% in the second.

On the three samples tested with a third portion of benzene, the amount of material recovered was less than 0.01% of the sample.

It is evident that two series of immersions made a sharp separation between the easily extractable material, mostly wax, of the surface and outer layers and the true oil from the interior of the grain.

After completion of the analysis for vitamins, oil, and nitrogen, composite samples of bran and of germ were made by combining all the remaining portions of these fractions. The results of starch and ash determinations on these samples do not represent the average composition of the five varieties, since the varieties did not contribute equally to the composite, but are indicative of the amounts of these constituents present.

Vitamin assays were made by microbiological methods. Riboflavin was determined by the method of Snell and Strong (12) following hydrolysis of the sample in 0.1 *N* hydrochloric acid at 120°C. for 30 minutes and filtration after neutralization to pH 6.8. Pantothenic acid was determined by the method of Skeggs and Wright (11). Biotin and pyridoxine were liberated by autoclaving at 120°C. for 30 minutes in the presence of 5 *N* sulfuric acid; biotin was determined in the neutralized filtrates by the method of Wright and Skeggs (16). The same filtrates were assayed for pyridoxine by the method of Atkin *et al.* (2).

### Discussion

The physical analysis and composition of the whole grain and the fractions are given in Table I. The proportion of endosperm in the sorghum varieties separated ranged from 80.0 to 84.6%; the germ from 7.8 to 12.1%; and the bran 7.3 to 9.3%. These results are remarkably similar to those from the 11 samples of corn reported by Earle, Curtis, and Hubbard (6) which ranged from 79.7 to 83.5% endosperm, 10.2 to 14.1% germ, and 4.4 to 6.2% bran, and about 1% tip cap, and are in good agreement with the work of Bidwell (3) and Bidwell, Bopst, and Bowling (4).

In general, sorghum grain and its fractions closely resemble corn in the proportions of starch, protein, oil, and ash. Protein and starch are about 2% higher in the whole sorghum grain than in corn. In the



germ, the oil content is about 6% lower and the starch 5% higher than in corn germ. Microscopic examination of the separated germ fraction showed that some of the starch, perhaps a third, resulted from incomplete separation of the endosperm. Bran is likewise high in starch but,

TABLE I  
COMPOSITION OF SORGHUM FRACTIONS AND WHOLE GRAIN  
(MOISTURE-FREE BASIS)

| Fraction and variety | Per cent of whole kernel | Chemical composition |         |           |                  |                   | Vitamin content |                          |                 |        |                 |
|----------------------|--------------------------|----------------------|---------|-----------|------------------|-------------------|-----------------|--------------------------|-----------------|--------|-----------------|
|                      |                          | Ash                  | Protein | Crude wax | Oil <sup>1</sup> | Starch            | Niacin          | Panto-<br>themic<br>acid | Ribo-<br>flavin | Biotin | Pyri-<br>doxine |
|                      |                          | %                    | %       | %         | %                | %                 | µg./g.          | µg./g.                   | µg./g.          | µg./g. | µg./g.          |
| Whole Grain          |                          |                      |         |           |                  |                   |                 |                          |                 |        |                 |
| Westland             |                          | 1.67                 | 13.2    | 0.29      | 3.5              | 72.3              | 47.8            | 15.5                     | 1.4             | 0.22   | 6.2             |
| Midland              |                          | 1.57                 | 12.0    | .31       | 3.6              | 74.5              | 43.9            | 9.2                      | 1.3             | .19    | 4.9             |
| Cody                 |                          | 1.68                 | 12.4    | .31       | 3.2              | 74.3              | 68.0            | 9.2                      | 1.1             | .18    | 4.2             |
| Pink Kafir           |                          | 1.67                 | 12.3    | .44       | 3.9              | 73.0              | 38.8            | 9.0                      | 2.0             | .23    | 4.2             |
| Martin               |                          | 1.57                 | 11.5    | .24       | 3.7              | 75.1              | 28.1            | 9.0                      | .8              | .20    | 4.0             |
| Average              |                          | 1.65                 | 12.3    | 0.32      | 3.6              | 73.8              | 45.3            | 10.4                     | 1.3             | 0.20   | 4.7             |
| Endosperm            |                          |                      |         |           |                  |                   |                 |                          |                 |        |                 |
| Westland             | 81.1                     | 0.44                 | 13.0    |           | 0.6              | 82.2              | 42.1            | 10.9                     | 1.1             | 0.11   | 4.7             |
| Midland              | 84.1                     | .39                  | 12.0    |           | .6               | 83.0              | 44.6            | 7.8                      | 1.3             | .10    | 4.3             |
| Cody                 | 84.6                     | .41                  | 12.3    |           | .8               | 83.0              | 70.5            | 9.5                      | .9              | .11    | 3.7             |
| Pink Kafir           | 80.0                     | .30                  | 12.8    |           | .4               | 83.2              | 34.6            | 6.8                      | .7              | .13    | 3.6             |
| Martin               | 81.7                     | .30                  | 11.2    |           | .4               | 81.3              | 26.9            | 8.6                      | .5              | .11    | 3.8             |
| Average              | 82.3                     | 0.37                 | 12.3    |           | 0.6              | 82.5              | 43.7            | 8.7                      | 0.9             | 0.11   | 4.0             |
| Germ                 |                          |                      |         |           |                  |                   |                 |                          |                 |        |                 |
| Westland             | 9.6                      |                      | 19.1    |           | 27.3             |                   | 105.5           | 51.1                     | 3.8             | 0.53   | 8.2             |
| Midland              | 8.5                      |                      | 19.1    |           | 30.6             |                   | 69.5            | 25.9                     | 4.3             | .70    | 7.6             |
| Cody                 | 7.8                      |                      | 18.0    |           | 28.6             |                   | 108.3           | 28.2                     | 3.8             | .54    | 7.2             |
| Pink Kafir           | 12.1                     |                      | 19.2    |           | 27.1             |                   | 70.5            | 27.1                     | 3.4             | .46    | 6.7             |
| Martin               | 11.0                     |                      | 19.0    |           | 26.9             |                   | 49.8            | 28.7                     | 4.0             | .60    | 6.5             |
| Average              | 9.8                      | 10.36 <sup>2</sup>   | 18.9    |           | 28.1             | 13.4 <sup>2</sup> | 80.7            | 32.2                     | 3.9             | 0.57   | 7.2             |
| Bran                 |                          |                      |         |           |                  |                   |                 |                          |                 |        |                 |
| Westland             | 9.3                      |                      | 6.8     |           | 3.7              |                   | 43.4            | 12.0                     | 3.6             | 0.30   | 4.9             |
| Midland              | 7.4                      |                      | 7.5     |           | 5.3              |                   | 53.8            | 8.4                      | 4.8             | .37    | 5.1             |
| Cody                 | 7.6                      |                      | 7.6     |           | 4.9              |                   | 62.6            | 11.3                     | 4.7             | .34    | 4.0             |
| Pink Kafir           | 7.9                      |                      | 6.4     |           | 6.0              |                   | 32.8            | 8.8                      | 3.9             | .42    | 4.0             |
| Martin               | 7.3                      |                      | 5.2     |           | 4.4              |                   | 27.4            | 9.3                      | 2.8             | .31    | 3.9             |
| Average              | 7.9                      | 2.02 <sup>2</sup>    | 6.7     |           | 4.9              | 34.6 <sup>2</sup> | 44.0            | 10.0                     | 4.0             | 0.35   | 4.4             |

<sup>1</sup> Oil in bran and whole grain includes wax.

<sup>2</sup> Analysis of composite, not true average.

in this case, microscopic examination showed that under the tempering and soaking conditions used, the bran separated within the starchy mesocarp. Accordingly, the bran consisted of the cuticle, epidermis, hypoderm, and the major portion of the mesocarp. The innermost

fragments of the mesocarp, the nucellar layer, and aleurone remained with the endosperm fraction (13, 15). This is in contrast to corn, in which the microscope shows no starch within the bran cells. Sorghum bran is higher in oil (petroleum ether extract) than corn bran, but the extract consists mostly of wax rather than oil. The extract is semi-solid at room temperature and can be processed to give a hard wax (9).

TABLE II  
PROPORTION OF THE TOTAL OF THE INDICATED CONSTITUENT EXISTING IN  
THE SPECIFIED FRACTION (CALCULATED FROM TABLE I)

| Fraction and variety | Constituents      |         |                  |                   |        |                          |                 |        |                 |
|----------------------|-------------------|---------|------------------|-------------------|--------|--------------------------|-----------------|--------|-----------------|
|                      | Ash               | Protein | Oil <sup>1</sup> | Starch            | Niacin | Panto-<br>thenic<br>acid | Ribo-<br>flavin | Biotin | Pyri-<br>doxine |
|                      | %                 | %       | %                | %                 | %      | %                        | %               | %      | %               |
| Endosperm            |                   |         |                  |                   |        |                          |                 |        |                 |
| Westland             |                   | 81.1    | 14.1             |                   | 70.7   | 59.5                     | 56.3            | 52.6   | 75.3            |
| Midland              |                   | 82.2    | 14.4             |                   | 79.3   | 70.0                     | 60.9            | 49.4   | 78.0            |
| Cody                 |                   | 84.0    | 20.6             |                   | 81.9   | 72.3                     | 54.2            | 57.4   | 78.5            |
| Pink Kafir           |                   | 78.3    | 7.9              |                   | 71.3   | 57.6                     | 43.4            | 53.6   | 71.5            |
| Martin               |                   | 78.7    | 9.1              |                   | 74.6   | 64.7                     | 37.6            | 50.6   | 75.5            |
| Average              | 20.6 <sup>2</sup> | 80.9    | 13.2             | 94.4 <sup>2</sup> | 75.6   | 64.8                     | 50.5            | 52.7   | 75.8            |
| Germ                 |                   |         |                  |                   |        |                          |                 |        |                 |
| Westland             |                   | 14.1    | 75.9             |                   | 21.0   | 33.0                     | 22.9            | 30.7   | 15.6            |
| Midland              |                   | 13.2    | 74.4             |                   | 12.5   | 23.4                     | 19.9            | 34.6   | 13.9            |
| Cody                 |                   | 11.3    | 68.0             |                   | 11.6   | 19.9                     | 20.8            | 26.5   | 13.9            |
| Pink Kafir           |                   | 17.8    | 80.5             |                   | 22.0   | 35.0                     | 32.5            | 29.2   | 20.5            |
| Martin               |                   | 18.0    | 82.0             |                   | 18.6   | 29.1                     | 42.5            | 36.8   | 17.5            |
| Average              | 68.6 <sup>2</sup> | 14.9    | 76.2             | 1.8 <sup>2</sup>  | 17.1   | 28.1                     | 27.7            | 31.6   | 16.3            |
| Bran                 |                   |         |                  |                   |        |                          |                 |        |                 |
| Westland             |                   | 4.8     | 10.0             |                   | 8.3    | 7.5                      | 20.8            | 16.7   | 9.1             |
| Midland              |                   | 4.6     | 11.2             |                   | 8.2    | 6.6                      | 19.2            | 15.9   | 8.1             |
| Cody                 |                   | 4.7     | 11.4             |                   | 6.5    | 7.8                      | 25.0            | 16.1   | 7.6             |
| Pink Kafir           |                   | 3.9     | 11.6             |                   | 6.7    | 7.4                      | 24.1            | 17.2   | 8.0             |
| Martin               |                   | 3.3     | 8.9              |                   | 6.8    | 6.2                      | 19.9            | 12.6   | 7.0             |
| Average              | 10.8 <sup>2</sup> | 4.0     | 10.6             | 3.8 <sup>2</sup>  | 7.3    | 7.1                      | 21.8            | 15.7   | 8.0             |

<sup>1</sup> Oil in bran includes wax.

<sup>2</sup> Calculated from analysis of composite sample.

Vitamin content varies much more among varieties than does the proportion of major constituents. Since the analyses of Tanner, Pfeiffer, and Curtis (14) show about twice the range in vitamin content reported here, it would seem that five samples are inadequate for a completely satisfactory picture. However, the general relationship between the composition of the fractions is probably shown satisfactorily by the five samples. For example, analysis of the germ of numerous varieties would probably extend the range of vitamin content

found in the germ, but would not change the conclusion that the germ is the richest of the fractions and contains in general a concentration from two to five times as high as that of the endosperm and bran. Riboflavin in bran is exceptionally high, equaling that in germ. Niacin, pantothenic acid, and pyridoxine occur in the bran and endosperm in essentially the same concentration.

Table II shows the proportion of each constituent which occurs in the various fractions. Five samples are probably sufficient also to give a satisfactory representation of this distribution.

#### Acknowledgment

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## Cereal Chemistry

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**General.** From January 1, 1948, an abstract will be printed at the beginning of each paper instead of a summary at the end, references will be numbered to provide the option of citing by number only, and date of receipt, author's connections, etc., will be shown in footnotes. Except on these points, authors will find the last volume of *Cereal Chemistry* a useful guide to acceptable arrangements and styling of papers. "On Writing Scientific Papers for Cereal Chemistry" (*Trans. Am. Assoc. Cereal Chem.* 6: 1-22, 1948) amplifies the following notes.

Authors should submit two copies of the manuscript, typed double spaced with wide margins on 8½ by 11 inch white paper, and all original drawings or photographs for figures. If possible, one set of photographs of figures should also be submitted. Originals can then be held to prevent damage, and the photographs can be sent to reviewers.

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**Tables.** Data should be arranged to facilitate the comparisons readers must make. Tables should be kept small by breaking up large ones if this is feasible. Only about eight columns of tabular matter can be printed across the page. Authors should omit all unessential data such as laboratory numbers, columns of data that show no significant variation, and any data not discussed in the text. A text reference can frequently be substituted for columns containing only a few data. The number of significant figures should be minimized. Box and side heading should be kept short by abbreviating freely; unorthodox abbreviations may be explained in footnotes, but unnecessary footnotes should be avoided. Leader tables without a number, main heading, or ruled lines are often useful for small groups of data.

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## BETA-AMYLASE ACTIVITIES OF BARLEY, WHEAT, AND RYE<sup>1</sup>

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### ABSTRACT

Three fractions of beta-amylase are thought to be present in ungerminated barley, wheat and rye, the common cereals of the botanical tribe Hordeae. One is water-soluble, the second is soluble in dilute salt solutions, and the third is made available by the action of proteolytic enzymes, such as papain.

Studies of the comparative amounts of these fractions were made, chiefly on barley and wheat, and were supplemented with data from the literature. Ungerminated grain, germinating grain, and malt were each extracted at 20°C. with water, salt solution, and papain solution respectively, for from two hours to three days, and the beta-amylase activity of the extracts determined.

Maximum beta-amylase activity of the water extract of the ungerminated grains was obtained within two to three days, and more quickly when the grains were germinated. When limited amounts of salts were added to the infusion, the activity of the extract increased. The amount of beta-amylase made available by such salt additions decreased as extraction progressed, and was negligible when extraction was completed. It also diminished as germination progressed. The maximum beta-amylase values to be reached in extraction were therefore not increased upon salt additions. The differential effects of salt additions were correlated with the ash contents of the cereals studied. It is concluded that the water-soluble and the salt-soluble activities constitute the "active" beta-amylase fraction, its water-soluble portion being the one which is made available by the water solution of salts contained in the grain.

The "inactive" fraction of the "total" beta-amylase activity is rendered water-soluble by proteolytic enzymes. This fraction was little affected by time and temperature of extraction, but decreased during germination while the "active" fraction increased.

The beta-amylase activity of a cereal malt is generally assumed to be preëxistent in the mature, ungerminated grain. Only a part of this activity, however, is readily available, or "free," in the water

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extract of an ungerminated cereal. The presence of substantial fractions of inactive beta-amylase appears to be common in wheat, barley, and rye (8, 21). While all cereals belong, botanically, to the Gramineae, or grass family, these three are in the tribe called Hordeaceae which does not include the other common cereals (18).

There is some controversy as to how the inactive portion of amylase is made available (11). Dull and Swanson (11) have suggested the term "bound" to designate the amylase fraction which is inactive due to physical causes, and "latent" for that rendered active by chemical reaction. Their opinion was that bound beta-amylase is released by salts from parts of the protein matrix, where it is adsorbed or occluded.

The use of papain in analytical methods for determining "total" beta-amylase activity is based on the fact that the latent fraction of the enzyme is made available by proteolytic enzymes. It is controversial whether this activation is the direct or indirect result of proteolysis, a reduction reaction involving the sulhydryl groups of the enzyme molecules (39), or results from the removal of inhibiting substances (19).

The present investigation was undertaken to secure further information concerning the comparative amounts of water-soluble, salt-soluble, and latent fractions of beta-amylase in ungerminated barley and wheat, in the germinating grains and in malts prepared from them.

### Materials and Methods

Samples of different types of barley, wheat, and rye were selected for the evaluation of the beta-amylase activities of the ungerminated grains, of these grains during germination, and of the final malts. The barleys were all of the six-rowed spring type used for commercial malting. Each sample lot of barley was designated by a serial number according to the origin, viz: North Dakota B-1 to B-11, South Dakota B-21 to B-25, Minnesota B-31 to B-38, and Montana B-41 to B-42. The wheats, W1 to W3, represented three different hard winter wheats; the rye samples were labeled R.

Ten grams of the conventionally ground samples of grain or malt were infused with 200 ml. distilled water or salt solution, with or without addition of two grams papain (Merck). The extractions were conducted at 20°C. over periods of 2, 22, 46, or 70 hours, and at 30°C. for 2 hours. Toluene was added to all extractions of more than 3 hours. Extractions at 40°C. were made in some instances but are not reported in this study because destructive effects on the beta-amylase activities became apparent, particularly in the presence of salts and of papain (3, 15).

The beta-amylase activities of the ungerminated grains were measured in the filtered extracts according to the conventional method for determining the diastatic power (Cereal Laboratory Methods, 5th ed., 1947). Alpha-amylase activities were disregarded because the corresponding saccharifying activities were below the margin of analytical error of the diastatic power evaluation, even in the presence of salts or papain.

For the germinated wet grains, which were dried *in vacuo*, and for the kiln-dried malts, both the diastatic power or total saccharifying activity and the alpha-dextrinizing activity were measured in each extract. The latter determination was by the method of Olson, Evans, and Dickson (29), but the equivalent saccharifying activities were averaged from the values given by these authors and those published by Ehrnst and Lucht (13), which differ somewhat in the lower and higher brackets of activities. The beta-amylase activities were calculated as the difference between the total and the alpha-saccharifying activities, and expressed in terms of maltose equivalent divided by 4 ( $^{\circ}$ L.).

### Results

*Influence of Salt- and Papain-Concentrations on the Availability of Beta-Amylase from Ungerminated Grains.* It has been reported for wheat (10, 20) and for barley (11) that the amount of beta-amylase extracted increases with the concentration of neutral salt present, but reaches a maximum at approximately 0.1 to 0.2 *N* salt concentration. In these experiments the salt used amounted to about 12 to 15% of the weight of the grain. Salts present in greater amounts may exert detrimental effects either by depressing the activity of the solution (14) or by acting as precipitating ("salting out") agents (30, 31).

When increasing quantities of proteolytic enzymes such as papain, trypsin, rennet (7, 8) or ficin (3) are added to the water infusions of the grains, increasing beta-amylase activities result in the extract, until the highest activity is reached in a given time of extraction. Twenty parts of commercial papain per hundred parts of grain are usually more than the quantity necessary to give solutions of maximum activity (10), but may exert slightly detrimental effects over longer periods of extraction, especially at higher temperatures (3).

The complementary effect of salt and papain is illustrated in Table I by values obtained on two very similar samples of hard wheat for which the influence of salt alone was pronounced. The data for the suboptimal papain concentrations are reproduced from the paper of Jozsa and Gore (20).

The salt effect exceeded that of papain at the low, suboptimal concentrations of the latter. Increasing papain quantities reduced this



difference and then progressively exceeded the activating influence of the salt until the highest activation of enzyme by papain was attained. The complementary effect of salt plus papain was pronounced at the lowest papain concentration compared with that of papain alone, but it diminished with increasing papain activation and disappeared as soon as the papain quantity became sufficient for maximum activation. Over prolonged periods of extraction, addition of salt to the papain-grain infusion resulted in lower beta-amylase values than with papain alone.

*Influence of Time of Infusion on the Water-, Salt-, and Papain-Soluble Beta-amylase Activities of Ungerminated Grains.* Results obtained in the extraction of several barley samples with distilled water

TABLE I  
COMBINED INFLUENCE OF SALT AND PAPAIN ON THE AVAILABILITY OF  
BETA-AMYLASE IN EXTRACTING HARD WHEAT

| Duration of Ex-<br>traction at 20°C. | Extraction-Medium |                     |                |              |           | Differential Effects      |                             |              |
|--------------------------------------|-------------------|---------------------|----------------|--------------|-----------|---------------------------|-----------------------------|--------------|
|                                      | Water Distd.      | Salt without Papain | Papain         |              |           | Papain compared with Salt | Papain + Salt compared with |              |
|                                      |                   |                     | Ratio to Wheat | Without Salt | Plus Salt |                           | Salt Alone                  | Papain Alone |
| Hours                                | °L.               | °L.                 | %              | °L.          | °L.       | °L.                       | °L.                         | °L.          |
| 2 <sup>1</sup>                       | 44                | 102                 | 0.01           | 48           | 103       | -54                       | + 1                         | +55          |
| 2 <sup>1</sup>                       | 44                | 102                 | 0.02           | 58           | 105       | -44                       | + 3                         | +47          |
| 2 <sup>1</sup>                       | 44                | 102                 | 0.03           | 72           | 115       | -30                       | +13                         | +43          |
| 2 <sup>1</sup>                       | 44                | 102                 | 0.10           | 90           | 120       | -12                       | +18                         | +30          |
| 2 <sup>1</sup>                       | 44                | 102                 | 0.25           | 119          | 147       | +17                       | +45                         | +28          |
| 2                                    | 38                | 98 <sup>2</sup>     | 20             | 176          | 178       | +78                       | +80                         | + 2          |
| 22                                   | 50                | 123 <sup>2</sup>    | 20             | 198          | 196       | +75                       | +73                         | - 2          |
| 46                                   | 62                | 128 <sup>2</sup>    | 20             | 203          | 195       | +75                       | +67                         | - 8          |

<sup>1</sup> Extraction results taken from the paper of Jozsa and Gore (20) with the kind permission of the publishers of Industrial and Engineering Chemistry; 0.03 *N* NaCl or 2% of wheat meal in 1:10 suspension.

<sup>2</sup> 0.1 *N* NaCl or 12% of wheat meal in 1:20 suspension (Wheat W-1).

without and in the presence of either salt or papain are reported in Table II. The figures are representative of similar experiments made on a greater number of different sample lots of spring barleys. Results reproduced from the paper of Davidson (9) are added because they refer to winter barleys of low diastatic power, treated under conditions of grain and salt concentrations which differed from those employed in our experiments.

The water-, salt-, and "total" papain-soluble activities of the spring barleys increased greatly during the first day of infusion at 20°C., they continued to rise somewhat to a maximum during a second, but showed practically no change on a third day. This is in agreement with similar

experiments reported in the literature (3, 27). Besides sample differences, the fineness of the grain meal influence such results (4). With Davidson's winter barleys the increases of the activities with time of extraction were less pronounced.

The activating effect of salt additions to infusions of spring barleys was small during the first hours of the extraction, and nearly disappeared in the longer extractions. Sodium and potassium chlorides,

TABLE II  
INFLUENCE OF DURATION OF INFUSION (AT 20°C.) ON THE AVAILABILITY OF BETA-AMYLASE IN WATER-, SALT-, AND PAPAIN-EXTRACTIONS OF BARLEY GRAIN

| Barley Sample    | 2 Hours      |      |        | 1 Day        |      |        | 2 Days       |      |        | 3 Days       |      |        |
|------------------|--------------|------|--------|--------------|------|--------|--------------|------|--------|--------------|------|--------|
|                  | Distd. Water | Salt | Papain | Distd. Water | Salt | Papain | Distd. Water | Salt | Papain | Distd. Water | Salt | Papain |
|                  | °L.          | °L.  | °L.    | °L.          | °L.  | °L.    | °L.          | °L.  | °L.    | °L.          | °L.  | °L.    |
| I <sup>1</sup>   |              |      |        |              |      |        |              |      |        |              |      |        |
| B-1              | 57           | 59   | 111    | 116          | 114  | 161    | —            | —    | —      | —            | —    | —      |
| B-3              | 69           | 76   | 120    | 111          | 116  | 160    | 123          | 126  | 168    | —            | —    | —      |
| B-6              | 80           | 84   | 130    | 122          | 130  | 175    | 133          | 136  | 180    | —            | —    | —      |
| B-21             | 69           | 76   | 125    | 108          | 109  | 156    | —            | —    | —      | —            | —    | —      |
| B-23             | 86           | 89   | 146    | 129          | 127  | 178    | —            | —    | —      | —            | —    | —      |
| B-25             | 93           | —    | 159    | 153          | —    | 218    | 176          | —    | 223    | —            | —    | —      |
| B-31             | 65           | 70   | 113    | 112          | 114  | 149    | 120          | 121  | 154    | —            | —    | —      |
| B-41             | 101          | 108  | 209    | 156          | 158  | 249    | 178          | 180  | 268    | —            | —    | —      |
| B-11             | 88           | 95   | 160    | 143          | 148  | 202    | 150          | 156  | 206    | 149          | 155  | 206    |
| B-11             | 88           | 98*  | 160    | 143          | 144* | 202    | 150          | 149* | 206    | 149          | 151* | 206    |
| II <sup>2</sup>  |              |      |        |              |      |        |              |      |        |              |      |        |
| B-11             | 87           | 93   | 147    | 141          | 152  | 207    | 150          | 156  | 206    | 153          | 163  | 206    |
| B-11             | 87           | 94*  | 147    | 141          | 144* | 207    | 150          | 155* | 206    | 153          | 152* | 206    |
| III <sup>3</sup> |              |      |        |              |      |        |              |      |        |              |      |        |
| B-a              | 31           | —    | 59     | 36           | —    | 62     | 48           | —    | 74     | 49           | —    | 74     |
| B-b              | 41           | 25   | 71     | 47           | 29   | 75     | —            | —    | —      | 57           | 33   | 86     |

\* Figures marked with asterisk refer to extractions in presence of KCl, all others to those in presence of NaCl.

<sup>1</sup> Spring barleys; grain infusion 1 part to 20 parts of water; salt in 0.1 N solution equivalent to 12% of the grain; papain equivalent to 20% of the weight of the grain.

<sup>2</sup> Spring barley; grain infusion 1 part to 30 parts water; salt in 0.17 N solution equivalent to 30% of the weight of the grain; papain added in amount equivalent to 15% of the weight of the grain.

<sup>3</sup> Winter barleys; extraction results reproduced from the paper of Davidson (9), obtained under infusion conditions identical to those described in footnote 2 (above).

compared on equimolar bases, gave similar results, but some significant differential salt effects on the availability of beta-amylase have been reported (11)<sup>4</sup>. According to Davidson's reports, salt additions resulted in lower beta-amylase values in the case of winter barleys. The lower values were not due to the difference in the extraction conditions, as shown by comparative experiments conducted with spring barleys, given in Table II under heading II.

<sup>4</sup> See Discussion. Sodium-bicarbonate was found to give extraction results similar to but always slightly higher than those obtained with sodium chloride; this is, however, in line with the fact that bicarbonate ion has a stimulating effect upon the beta-amylase activity in water solution (14).

The beta-amylase activities of wheat show trends which are basically similar to those described for barley. In the case of wheat also, the type of grain greatly influences the beta-amylase activity (9, 20, 33, 34). Extraction results for some examples of wheat and rye are given in Table III.

In these examples, the rate at which the water-soluble fraction became available was slow compared with that of the spring barleys, and solubilization still continued after two days of infusion at 20°C.

The salt additions, however, increased the extraction of the beta-amylase to a much higher degree than for barley in the initial periods of the infusion, but the salt effect diminished and finally disappeared

TABLE III

INFLUENCE OF DURATION OF INFUSION (AT 20°C.) ON THE AVAILABILITY OF BETA-AMYLASE IN WATER-, SALT-, AND PAPAIN-EXTRACTIONS OF WHEAT AND RYE GRAINS

| Grain Sample     | 2 <sup>1</sup> or 3 <sup>2</sup> Hours |      |        | 1 Day        |      |        | 2 Days       |      |        | 3 Days       |      |        |
|------------------|----------------------------------------|------|--------|--------------|------|--------|--------------|------|--------|--------------|------|--------|
|                  | Distd. Water                           | NaCl | Papain | Distd. Water | NaCl | Papain | Distd. Water | NaCl | Papain | Distd. Water | NaCl | Papain |
|                  | °L.                                    | °L.  | °L.    | °L.          | °L.  | °L.    | °L.          | °L.  | °L.    | °L.          | °L.  | °L.    |
| <i>Wheat</i>     |                                        |      |        |              |      |        |              |      |        |              |      |        |
| W-1 <sup>1</sup> | 38                                     | 98   | 176    | 50           | 123  | 198    | 62           | 128  | 203    | —            | —    | —      |
| W-3 <sup>1</sup> | 53                                     | 91   | 151    | 64           | 131  | 185    | 135          | 141  | 188    | 150          | 141  | 185    |
| W-a <sup>2</sup> | 45                                     | —    | 90     | 44           | —    | 90     | 45           | —    | 89     | 47           | —    | 87     |
| W-b <sup>2</sup> | 54                                     | 75   | 104    | 54           | 80   | 102    | 59           | —    | 103    | 61           | 78   | 103    |
| <i>Rye</i>       |                                        |      |        |              |      |        |              |      |        |              |      |        |
| R-1 <sup>1</sup> | 66                                     | 71   | 94     | 78           | 79   | 96     | 79           | 82   | 94     | 81           | 83   | 96     |
| R-a <sup>2</sup> | 66                                     | —    | 85     | 71           | —    | 89     | 77           | —    | 90     | 83           | —    | 94     |
| R-b <sup>2</sup> | 89                                     | 84   | 111    | 96           | 92   | 112    | 102          | —    | 114    | 102          | 93   | 109    |

<sup>1</sup> Grain infusion 1 part to 20 parts of water; salt in 0.1 *N* solution equivalent to 12% of the grain; papain added in amount equivalent to 20% of the weight of the grain.

<sup>2</sup> Extraction results reproduced from the paper of Davidson (9): Infusions of 1 part grain to 33 parts water; salt in 0.17 *N* solution equivalent to 30% of the weight of the grain; papain added in amount equivalent to 15% of the weight of the grain.

when the extraction was sufficiently prolonged to give the highest level of the water-soluble activity. In the latter case, the added salt sometimes had a slightly depressing action.

The effects on rye are similar in principle, and are also subject to differences dependent upon the type of grain (7, 9, 21).

The papain-activated beta-amylase fractions, calculated as the difference between the "total" and the respective water- and salt-soluble activities, are recorded in Fig. 1, for three samples of barley with widely different diastatic activities, for one hard wheat and one rye.

The water-insoluble fractions of barley and rye decreased pronouncedly during the first day of extraction, but to a lesser degree on the following days. The corresponding effects on wheat were quite

inconsistent during the initial periods of infusion; strong decrease of this fraction occurred, however, with progressing-extraction. Calculation of these papain-activated fractions from data found in previous publications confirms these findings.

The salt-insoluble fractions were much less affected by the time of extraction; they decreased but slightly in the initial periods and still less with more complete extractions. The similarity observed in this respect for barley and rye as well as for wheat appears to be significant.

Experiments conducted comparatively at 20°C. and at 30°C. showed the influence of higher extraction temperature to be in accordance with that of increased duration at the same temperature.

*Influence of Germination on the Beta-Amylase Activity of Barley and Wheat.* The "free" beta-amylase activity, as determined conventionally in two to three hour infusions, increases greatly during the

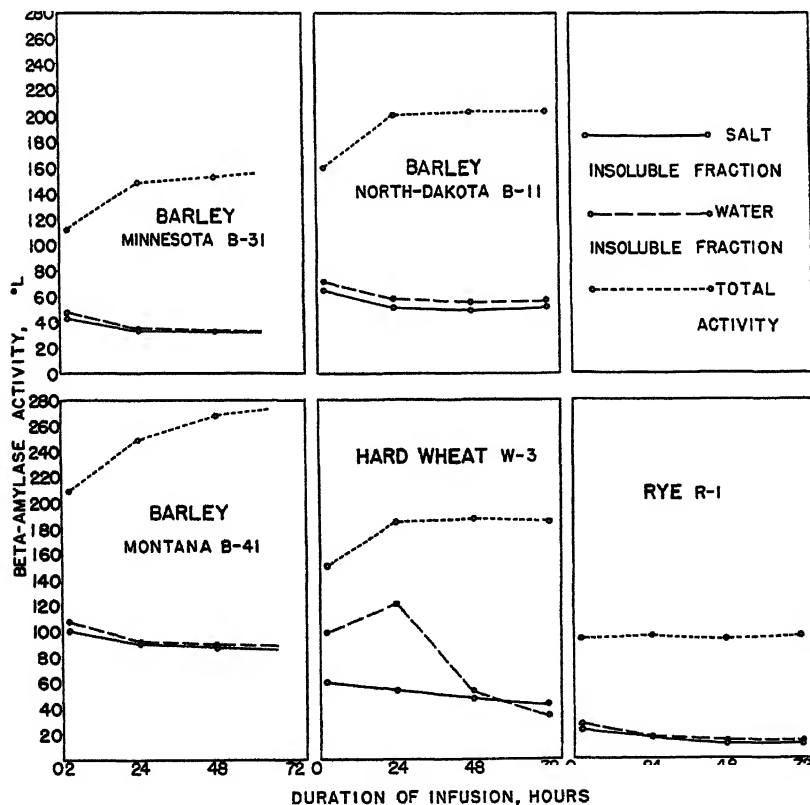


FIG. 1. Effect of the time of infusion on the water- and salt-soluble beta-amylase fractions as activated by papain in ungerminated barley, wheat, and rye. Infusion at 20°C. of one part grain for 20 parts water; salt effect of 0.1 N sodium chloride. Beta-amylase activities expressed in maltose-equivalents divided by four.

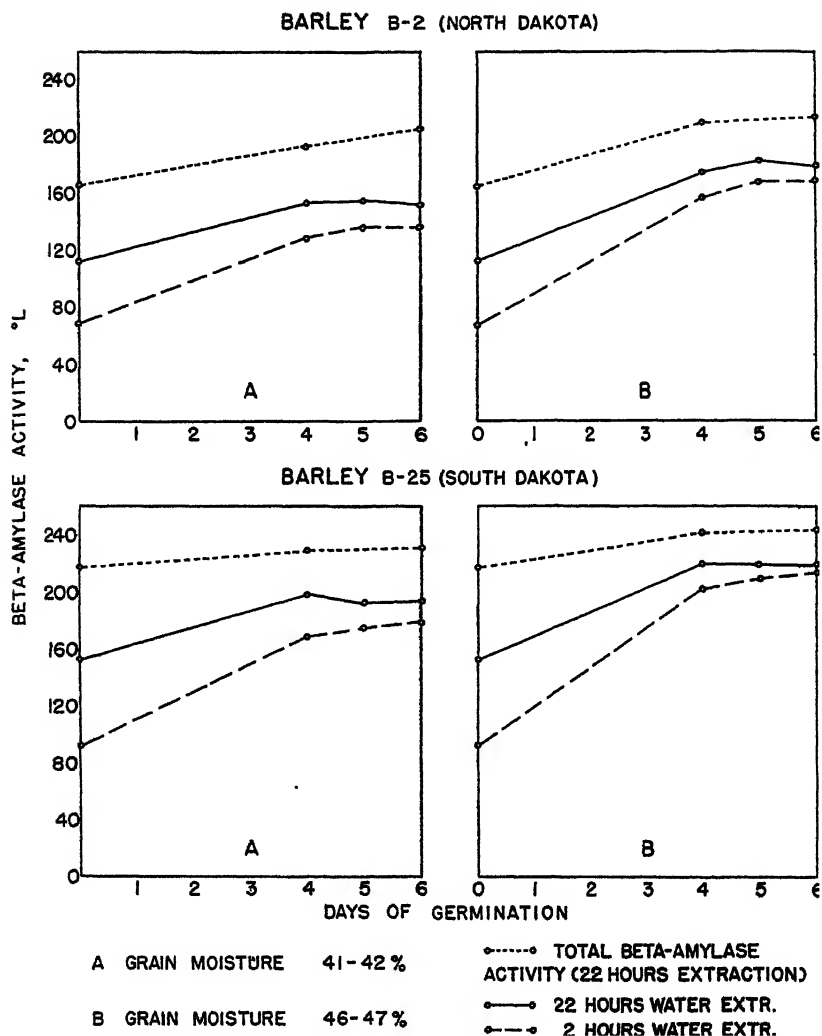


FIG. 2. Development of the water-soluble beta-amylase activities, measured after 2- and 22-hour infusions, during germination of commercial barleys. Germination at 16°C., moisture content of the germinating grains maintained at different levels, as indicated. Beta-amylase activities expressed in maltose-equivalents divided by four.

germination of the grains, much more than does the "total" activity (6, 16, 21, 22, 24, 26). It was of interest to compare the results of short and of prolonged grain infusions during germination. Two different samples of barley served each for 16 malting operations in which groups of four were conducted under similar conditions. For each group, the grain moisture was maintained on a different level, constant throughout the germination. The mean values obtained for the two

barleys on two of these groups of subsamples after four, five and six days of germination, are shown in Fig. 2.

The influence of duration of extraction upon the availability of water-soluble beta-amylase diminishes greatly with increasing length of germination, i.e., with the disaggregation of the inner kernel structure. Therefore, the "free" activity measured after 22-hour infusion increases during germination less than does the conventionally determined beta-amylase activity. The same is true for the "total" activity. A 22-hour infusion at 20°C, is practically sufficient for full availability of the free and the total beta-amylase activities of commercial barley malts.

TABLE IV  
INFLUENCE OF THE DURATION OF INFUSION ON THE AVAILABILITY  
OF THE WATER-SOLUBLE BETA-AMYLASE ACTIVITIES  
OF KILN-DRIED MALTS

| Sample                      | Infusion 20°C., 2 hrs. |                |         | Infusion 20°C., 22 hrs. |               |         | Increase from 2 to 22 hrs. |               |        |
|-----------------------------|------------------------|----------------|---------|-------------------------|---------------|---------|----------------------------|---------------|--------|
|                             | Raw Grain              | Malt dried at: |         | Raw Grain               | Malt dried at |         | Raw Grain                  | Malt dried at |        |
|                             |                        | 50°C           | 72°C.   |                         | 50°C          | 72°C.   |                            | 50°C.         | 72°C.  |
| B-5                         | °L. 77                 | °L. 139        | °L. 116 | °L. 119                 | °L. 154       | °L. 134 | °L. 42                     | °L. 15        | °L. 18 |
| B-8                         | 99                     | 179            | 140     | 141                     | 201           | 143     | 42                         | 22            | 3      |
| B-22                        | 77                     | 149            | 108     | 128                     | 171           | 127     | 51                         | 22            | 19     |
| B-24                        | 90                     | 167            | 131     | 130                     | 185           | 144     | 40                         | 18            | 13     |
| B-32                        | 83                     | 161            | 114     | 136                     | 168           | 132     | 53                         | 7             | 18     |
| B-35                        | 100                    | 176            | 139     | 143                     | 183           | 154     | 43                         | 7             | 15     |
| Mean values for the barleys |                        |                |         |                         |               |         | 45                         | 15            | 14     |
| W-1                         | 98                     | 127            | —       | 123                     | 143           | —       | 25                         | 16            | —      |

The drying conditions of the malts and consequent losses in activity have little bearing upon these results. This is shown in Table IV for malts which were dried at two different temperature levels and compared with the respective ungerminated grains.

The salt effect on malt extractions is illustrated in Table V.

The small stimulating influence of salt upon availability of the enzyme, noted for the ungerminated spring barleys, practically disappeared upon germination. In wheat, the strong salt effect observed for the ungerminated grain became markedly reduced upon germination, but still remained appreciable (14, 15). The availability of the salt-soluble beta-amylase increases during germination, but to a lesser degree than that of the respective water-soluble fractions.

The papain-activated fractions of beta-amylase in both barley and

wheat malts, little affected by the conditions of infusion, were appreciably lower than in the respective ungerminated grains. Some examples are given in Table VI for commercial malts of 5 to 6 days germination.

Comparisons of the activities were based upon measurements obtained in two-day extractions of the ungerminated grains and one-day extractions of the malts. The papain-activated fractions decreased progressively in amount during germination; this is illustrated by sample B-2 which compares three stages of development of green malt. In commercial malts substantial amounts of the papain-activated

TABLE V  
INFLUENCE OF SALT ADDITIONS ON THE BETA-AMYLASE  
ACTIVITIES IN THE EXTRACTION OF MALTS

| Sample                                                     | Infusion 20°C., 2 hrs. |      |                 | Infusion 30°C., 2 hrs. |      |          | Infusion 20°C., 22 hrs. |      |                 |
|------------------------------------------------------------|------------------------|------|-----------------|------------------------|------|----------|-------------------------|------|-----------------|
|                                                            | Water                  | NaCl | Increase        | Water                  | NaCl | Increase | Water                   | NaCl | Increase        |
|                                                            | °L.                    | °L.  | °L.             | °L.                    | °L.  | °L.      | °L.                     | °L.  | °L.             |
| B-9                                                        | 109                    | 111  | 2               | 104                    | 110  | 6        | —                       | —    | —               |
| B-10                                                       | 165                    | 165  | 0               | 169                    | 170  | 1        | 176                     | 176  | 0               |
| B-26                                                       | 82                     | 84   | 2               | 80                     | 83   | 3        | —                       | —    | —               |
| B-32                                                       | 114                    | 115  | 1               | 115                    | 114  | - 1      | 132                     | 129  | - 3             |
| B-36                                                       | 118                    | 117  | - 1             | 119                    | 120  | 1        | 124                     | 125  | 1               |
| B-37                                                       | 114                    | 116  | 2               | —                      | —    | —        | 122                     | 122  | 0               |
| B-38                                                       | 105                    | 105  | 0               | —                      | —    | —        | 116                     | 116  | 0               |
| Mean values<br>of increases { on malts<br>on raw<br>grains |                        |      | 1               |                        |      | 2        |                         |      | 0               |
|                                                            |                        |      | 5 <sup>1</sup>  |                        |      | 3        |                         |      | 2 <sup>1</sup>  |
| W-1                                                        | 82                     | 127  | 45              | 85                     | 137  | 52       | 110                     | 143  | 33              |
| Increase on raw grain:                                     |                        |      | 60 <sup>2</sup> |                        |      |          |                         |      | 73 <sup>2</sup> |
| W-2                                                        | 46                     | 78   | 32              | 56                     | 80   | 24       | —                       | —    | —               |

<sup>1</sup> See Table II.

<sup>2</sup> See Table III.

fraction of the enzyme are present, but when germination is continued beyond usual practice, the beta-amylase becomes completely water-soluble as shown by previous workers for wheat (22) and for barley (38)<sup>6</sup>.

The "total" beta-amylase activity of commercial green malt is always higher than that of the respective ungerminated grain, compared on a weight basis. Losses in beta-amylase activity become noticeable after prolonged periods of germination (22), but are unusual in normal malting practice. The increases reported in Table VI can be

<sup>6</sup> Weichherz and Asmus (38) found the "total" diastatic power remaining slightly higher than the "free" activity, but they disregarded the papain effect upon the alpha-amylase activity (14).

TABLE VI  
COMPARISON OF BETA-AMYLASE ACTIVITIES OF MALTS AND OF  
THE RESPECTIVE RAW GRAINS

| Sample | Salt-Soluble Fractions <sup>1</sup> |                    | Total Activities <sup>2</sup> |                    |                          | Papain-Activated Fractions |                   |             |
|--------|-------------------------------------|--------------------|-------------------------------|--------------------|--------------------------|----------------------------|-------------------|-------------|
|        | Raw Grain                           | Green Malt         | Raw Grain                     | Green Malt         | Kilned Malt <sup>3</sup> | Raw Grain                  | Green Malt        | Kilned Malt |
|        | °L.                                 | °L.                | °L.                           | °L.                | °L.                      | °L.                        | °L.               | °L.         |
| B-2    | 122                                 | 153                | 168                           | 195                | —                        | 46                         | 42                | —           |
| B-2    | 122                                 | 177                | 168                           | 209                | —                        | 46                         | 32                | —           |
| B-2    | 122                                 | 186↓               | 168                           | 215↓               | —                        | 46                         | 29↓               | —           |
| B-3    | 126                                 | —                  | 168                           | —                  | 158*                     | 42                         | —                 | 30          |
| B-4    | 136                                 | —                  | 193                           | (210) <sup>4</sup> | 194                      | 57                         | —                 | 37          |
| B-5    | 132                                 | 174                | 180                           | 194                | 154*                     | 48                         | 20                | 14          |
| B-6    | 136                                 | —                  | 180                           | —                  | 167*                     | 44                         | —                 | 26          |
| B-7    | 149                                 | 186                | 191                           | 207                | 202                      | 48                         | 21                | 12          |
| B-8    | 154                                 | 206                | 213                           | 227                | 170*                     | 59                         | 21                | 27          |
| B-22   | 141                                 | 172                | 194                           | 195                | 132*                     | 53                         | 23                | 5           |
| B-24   | 143                                 | 176                | 182                           | 211                | 160*                     | 39                         | 35                | 16          |
| B-25   | 178                                 | 223                | 223                           | 244                | —                        | 45                         | 21                | —           |
| B-32   | 149                                 | 182                | 205                           | 215                | 148*                     | 56                         | 33                | 16          |
| B-34   | 136                                 | 179                | 177                           | 193                | 186                      | 41                         | 14                | 11          |
| B-35   | 156                                 | 196                | 202                           | 222                | 177*                     | 46                         | 26                | 23          |
| B-42   | 203                                 | —                  | 286                           | (310) <sup>4</sup> | 294                      | 83                         | —                 | 63          |
| W-1    | 128                                 | (159) <sup>4</sup> | 203                           | (219) <sup>4</sup> | 203                      | 75                         | (60) <sup>4</sup> | 60          |

<sup>1</sup> Activities measured after 46 hours infusion with 0.1 N NaCl solution for the raw grains, after 22 hours for the malts.

<sup>2</sup> Total activities extracted in presence of 20% papain of grain and malt; 46 hours for grains, 22 hours for malts.

<sup>3</sup> The malts marked with an asterisk (\*) are brewers, dried at final temperatures of 72°C., the others were dried at 50°C.

<sup>4</sup> Values are estimated.

explained by the fact that 6 to 9% of the dry matter is lost during the malting process; in a few cases the difference was found to be somewhat greater, but it must be considered that infusion of the ungerminated grain over periods of two days may not always give complete extraction and infusions continued beyond that duration may give low results, especially in presence of salts and of papain.

### Discussion

Intervarietal correlation between the total saccharifying power of barley and the salt-solubility of the protein fractions has been shown to exist (1). Increase in availability of the beta-amylase of barley by infusion with solutions of salts of various anions and in different concentrations is of a nature similar to such salt effects on the peptization of the grain proteins (11, 36).

It is known that "free" beta-amylase increases with the concentration of the flour mash (20), it is higher in whole wheat than in patent flour, and still greater in the low-grade flour portions, parallel with the increasing ash contents (5, 9, 37). Likewise the degree of peptization



of different wheat flours has been shown to be positively correlated with their ash contents (32), as are also the proteolytic activities (17). The availability of the latter in grain infusions is apparently also increased in the presence of salts (12, 23).

Similar differences in the water-solubility of beta-amylase should become apparent when different grain types are compared with regard to their ash content. Barley, for instance, may vary in its ash on a dry basis from 2.4 to 3.5% (Spring barleys have usually 2.5 to 3.0% ash, Winter varieties up to 3.5%). Wheat has normally less than 2% ash (hard varieties 1.4 to 2.1%); the ash of rye is intermediate between barley and wheat.

According to the experiments reported here, salts only slightly increase the beta-amylase activity of spring barleys and of rye, but effect a considerable increase in the case of hard wheats. Davidson (9), reported the beta-amylase activity of winter barleys pronouncedly depressed, that of rye only slightly, and that of wheat increased. This could mean that the soluble ash of the barleys and ryes was sufficient to release nearly all or all of the "bound" beta-amylase, whereas the wheats needed substantial supplementation by added salts to complete the liberation, unless the time of extraction was considerably extended.

As reported in the present study, prolonged infusion of grains and malts in distilled water led to increasing beta-amylase activity until a maximum was obtained. Salts added in limited amounts accelerated this solubilization more or less, but the maximum availability remained the same as could be reached in sufficiently long water infusions.

In the malting process, the soluble minerals apparently diffuse into the core of the kernel during the soaking of the grain, and can be expected to act upon bound beta-amylase similarly, though more slowly, than they do in the water infusions of the grain meals. This may be one of the reasons why beta-amylase is more readily made available by water as germination progresses. Similarly, the accelerating effect of salts noted in the infusions of the ungerminated grains, is reduced or disappears upon their germination.

Therefore, it seems unwarranted to make a fundamental distinction between water-soluble, or "free" beta-amylase, and the "bound" or salt-soluble enzyme. The conclusion is suggested that both portions are "active" though not immediately available in their entirety because adsorbed, enveloped, or otherwise physically bound to certain fractions of the protein matrix. This "active" beta-amylase is completely released upon action of salts in solution furnished partly or fully by the grain ash. Such release proceeds simultaneously with peptization of the protein complexes, which is not necessarily a protein hydrolysis (35).

The "inactive" or "latent" beta-amylase fraction should be considered as that portion of the "total" activity which can not be made available by water in the presence of adequate salt quantities. The "inactive" beta-amylase is present either in reversibly oxidized form or chemically bound to another protein molecule. It is rendered water-soluble by proteolytic enzymes acting either as such or as reducing agents involving the sulfhydryl groups, or both.

It is here reported that these "inactive" fractions are little affected when time or temperature of the grain infusions are increased. In fact, they diminish slightly under such conditions, probably due to proteolytic action of the grain enzymes which are present. Curves plotted for the "total" activities against time of infusion show nearly the same slope as those of the respective salt-soluble "active" fractions and the increasing availability of the latter determines predominantly that of the "total" papain-soluble activities. Salts added to the papain-water infusions have either no effect or a detrimental effect. Commercial papain preparations contain enough soluble mineral salts to supply the grain-water infusions with the necessary salt supplements (14). Heat-inactivated papain has been reported to increase the water-extracted beta-amylase fraction of barley (7). Myrbäck and Myrbäck (28) did not confirm this effect but this is not necessarily a contradiction; they measured after a 20-hour infusion at 30°C., thus after the activity had been already completely released with water, whereas Chrzaszcz (7) compared the activities after 14 hours at 20°C; see above Table II. Papain thus provides for the release of both the "active" and the "inactive" beta-amylase.

During germination the proteolytic activities of the cereals are known to increase greatly (2, 25), and this may cause the progressive decrease of the "inactive" beta-amylase fraction. The "active" fraction, in turn, increases correspondingly beyond that present in the ungerminated grains. This increase, however, is much smaller than that of the seemingly free activity measured on the basis of conventional water extractions.

It is of interest to note that these changes, which occur during germination, take a course opposite to that apparent during the ripening periods of the cereal grains, as shown for wheat by Schwimmer (34), who thought the progressive insolubilization of beta-amylase to be connected with the dehydration of the maturing grain and the formation of the final protein complexes.

The effects of salts and of papain on the availability of cereal alpha-amylase, discussed for barley and wheat malts by Erlich and Burkert (14), are apparently of a different nature than those on beta-amylase. However, when these activities are measured in their water extracts,

it has to be considered for both types of enzymes that any infusion of cereal grains or malts in distilled water is actually an extraction by a weak salt solution produced by the soluble ash minerals. This is particularly important when the release of the enzyme activity from the grain is highly sensitive to small changes in salt concentration. Such is the case for alpha-amylase of both barley and wheat malts and for beta-amylase of wheat grain and flour, of malted wheat flour and some commercial wheat malts, but to a lesser degree for ungerminated barley and rye; it is not the case for beta-amylase of barley malt.

The presence of salts and possibly other soluble compounds in commercial papain also influences the utilization of these preparations in analytical procedures. In the case of alpha-amylase, the action of papain is apparently due only to its soluble ash content, and the maximum effect is lower than that obtainable with adequate salt solutions. No chemical activation of an "inactive" alpha-amylase fraction can be observed, as common for the beta-amylase activities of wheat, barley and rye.

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## POLYOXYETHYLENE MONOSTEARATE AS AN ANTI-STALING ADJUNCT IN BREAD<sup>1,2</sup>

B. G. CARSON, L. F. MARNETT AND R. W. SELMAN

### ABSTRACT

Various methods for determining the physical changes taking place during the staling of bread baked with and without the addition of polyoxyethylene monostearate have been evaluated. Measurements of the decrease of crumb thickness indicate that the use of this adjunct markedly retards the firming of bread as it ages from 4 to 115 hours.

The crumbliness test for staling, the Katz method for measuring the decrease in soluble starches, the swelling power test, and the farinograph method of measuring the decrease in water absorptive capacity give anomalous results when bread contains polyoxyethylene monostearate. This failure to give measurements indicative of the age of the bread is attributed to the polyoxyethylene monostearate having reduced the hydrophilic characteristics of the starch so that the normal molecular alignments of starch chains with each other and with water fail to occur.

The only traditional staling rate measurements which appear to be of value are those dealing with changes in softness and possibly in crumbliness, but crumbliness appears to be more indicative of tenderness than of age.

Katz (10), who did such extensive work on the problem of bread staling during the period 1912-1939, devised many of the tests for staling in use today. Most of these have been modified and revised to make use of present-day equipment, but their essential character has remained unchanged. Since 1930 several reviews of these staling tests and their modifications have appeared in *Cereal Chemistry*. Mention should be made especially of the reviews by Platt (11), Cathcart (4), Alsberg (1), and Geddes and Bice (9).

This paper reports the results of applying the traditional staling tests to bread containing polyoxyethylene monostearate (POEMS). The studies reported show the effect of POEMS on (a) changes in compressibility or, as Bice and Geddes (3) prefer "changes in the softness of bread crumb," (b) changes in crumbliness as a measure of staling, (c) changes in crumbliness as a measure of tenderness, (d) changes in water absorptive power as a measure of staling and (e) changes in soluble starch content as a measure of staling. In addition the effect of POEMS on the wetting rate of bread crumb is reported.

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<sup>2</sup> Contribution from C. J. Patterson Company, Kansas City, Missouri.

### Changes in Compressibility

The most noticeable change taking place in bread as it becomes stale is the increase in firmness or the decrease in compressibility of the crumb.

Katz (10), Platt (11), Bailey (2), Combs (6), Straub (12), and many others have described compressimeters to be used in the measurement of staling rates. Two techniques are involved. Either the instrument determines the weight necessary to bring about a predetermined decrease in crumb thickness or it determines the decrease in thickness caused by addition of a given weight. In this paper the latter method is applied. In all instruments, the pressure is applied for a definite length of time. These methods have been discussed fully by Bice and Geddes (3).

For the various studies reported in this paper, all bread was baked by the same sponge-dough method, employing the following formula except where otherwise indicated:

| <i>Ingredient</i> | <i>Per Cent</i> | <i>Weight (gms.)</i> |
|-------------------|-----------------|----------------------|
| Flour             | 100             | 700 (14% moisture)   |
| Water             | 57-69           | 399-483              |
| Yeast             | 2.0             | 14.0                 |
| Yeast food        | 0.5             | 3.5                  |
| Milk              | 3.0             | 21.0                 |
| Sugar             | 5.0             | 35.0                 |
| Lard              | 2.0             | 14.0                 |
| Salt              | 2.0             | 14.0                 |

A 60% sponge with an absorption of 57% was used. All the yeast was dispersed in the sponge water prior to the addition of the flour. In mixing sponge ingredients, yeast food and malt (when needed) were added directly on top of the flour.

The sponge was mixed one minute at low speed, then one minute in second speed. To prevent excessive flour dust, the water and yeast solutions were poured into the mixing bowl before the other sponge ingredients were added. The sponge was placed in a metal trough and fermented four and one-half hours at 84°F. (29°C.) and 85% relative humidity.

The dough was mixed as follows: Dry ingredients and the remaining water were placed in the bowl and mixed at the lowest speed for three minutes. During the first minute the sponge was added in three or four approximately equal lumps. After low-speed mixing, the dough was mixed at second speed for the predetermined time. The mixing bowl was jacketed with ice water and chilled water was used as the dough water so that the dough came from the mixer at 80°F. (26.6°C.).

Immediately after mixing, the dough was again placed in the fermentation trough and a thermometer was inserted. After one minute

the temperature was recorded and the trough was returned to the fermentation cabinet for 35 minutes. At the end of this period the dough was removed and two 380 gm. pieces scaled from it. Each of the pieces was formed into a ball by a manual operation which was intended to simulate the action of the commercial rounder. The pieces were then dusted and allowed to stand at room temperature on a dusted board for ten minutes.

After this period, which corresponds to the overhead proof time employed in the commercial bake shop, each piece was molded in a conventional commercial molder, placed in a pan, numbered and proofed in a proof box at 102°–104°F. (39°–40°C.) and 80% relative humidity until the top surface of the dough was  $\frac{1}{2}$  in. above the top of the pan.

The doughs were then placed in an oven and baked at 425°F. (218°C.) for 25 minutes. Baked loaves were placed on a rack and cooled to 100°F. (38°C.) before being doubly wrapped and heat-sealed in moisture proof waxed paper.

The compressimeter used in these studies consisted of a metal square, 2.5 in. by 2.5 in. and approximately 0.5 in. thick, which was attached rigidly at right angles to a round metal rod graduated in tenths of an inch. The combined plate and scale weighed 300 gm. In use, the rod was passed through a 2-in. glass tube which acted as a guide and on which was etched a reference mark. The instrument was adjusted before use by placing a 1-in. block under the metal plate and setting the reference mark to coincide with the 1-in. mark on the scale.

With the aid of a miter box, a slice of bread 1 in. thick was cut from the center portion of a loaf. The bottom crust was cut away and with the aid of a second miter box a 2-in. square was cut from the center of the slice. Any square containing large holes or a core was rejected. The sample was placed on a flat surface directly under the compressimeter plate and the plate was lowered until its face just touched the surface of the crumb. The zero reading representing the crumb thickness was taken from the scale. The compressimeter was released for a period of 60 sec., after which a second reading was made. The difference between the first and second readings corresponds to the decrease in crumb thickness. This difference multiplied by 100 and divided by the original thickness gave the per cent decrease in thickness of the crumb.

In all determinations of crumb compressibility, bread containing 0.5% polyoxyethylene monostearate (flour basis), the stearic acid ester of polyethylene glycol and hereafter designated as POEMS, was compared with bread containing no POEMS but otherwise baked at the same time by the same formula from the same flour under the same conditions.

Compressibility data taken during a  $1\frac{1}{4}$  year period on a total of 179 samples of bread containing POEMS were averaged and the average compressibilities at 0.75, 1.75, 2.75, and 3.75 days are shown in Fig. 1. Several loaves, each containing a different sample of POEMS, were usually compared with a loaf containing a standard sample of POEMS and a loaf baked without the addition of POEMS. In all instances the POEMS was used at the 0.5% level. In all instances compressibilities were determined on three different days but not necessarily on consecutive days. The loaves were stored at room temperature, which, of course, varied from summer to winter and, to a lesser degree, from day to night.

All the curves in the literature have assumed the form of an equilateral hyperbola. The curves of Fig. 1 do not represent staling rates at any given temperature, but rather the average firming rates for

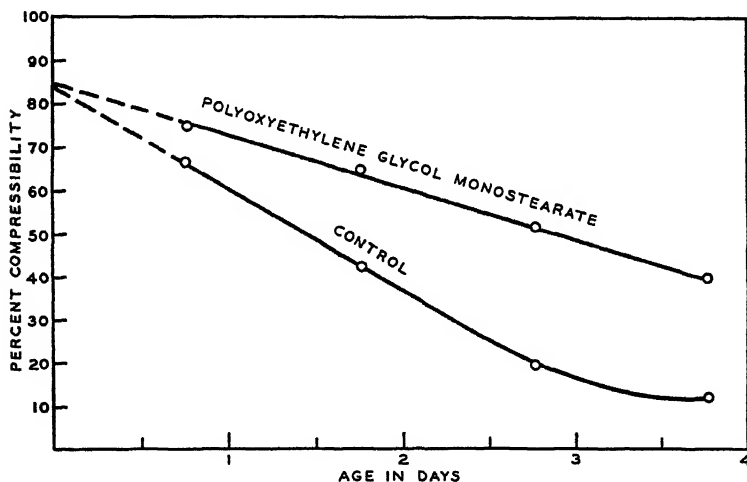


FIG. 1. The effect of age on compressibility

bread containing POEMS and bread baked without the adjunct. The curve for the control bread has a steeper slope than that for the bread containing POEMS. Extrapolation of each curve to zero time indicates that both types of bread have the same initial softness.

Compressibility tests were also made on bread baked with 0%, 0.25%, 0.5%, 1.0%, and 2.0% POEMS. All bread was doubly wrapped in moisture-proof paper and stored at room temperature, approximately 70°–74°F. Starting when the loaves had been out of the oven four hours, compressibilities were determined at intervals until the bread was 115 hours old.

The crumb recoveries at the 4-hour period were not good, e.g., the



elastic limits had been exceeded. During the first eight hours after bread came from the oven, changes in bread firmness took place so rapidly that experimental errors in testing were no doubt very high. The results represented graphically in Fig. 2 show that the use of POEMS definitely changed the firming rate. Bread containing 0.5% POEMS had about the same compressibility at 77 hours that control bread had at 35 hours.

*Effect of Increased Water on Compressibility.* It appeared desirable to design experiments which would indicate whether the retarded firming of bread containing POEMS is caused by its reaction with the starch, thereby preventing water from being bound through hydrogen

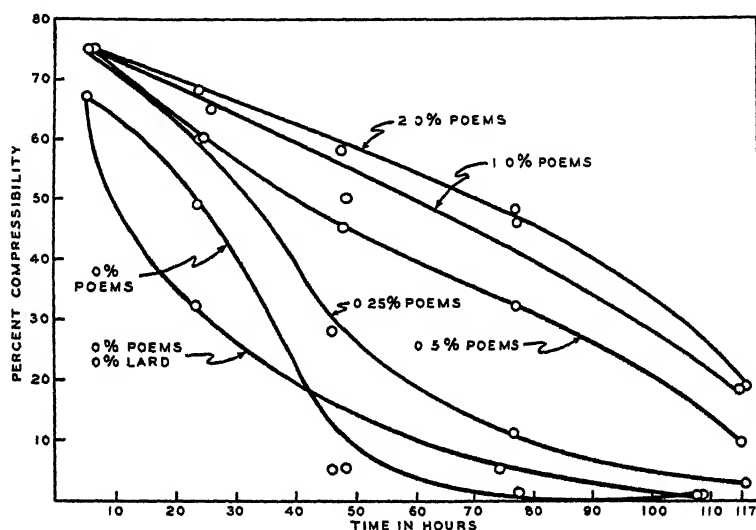


FIG. 2. Effect of polyoxyethylene monostearate on compressibility

bonding and thus leaving it free to exert a plasticizing effect on the crumb. In other words, could the same retarded firming be brought about by the use of additional water in the dough, assuming that this water is retained in the baked bread?

Three series of loaves were baked with and without 0.5% POEMS. In each series water was increased in 2% increments until the dough was baked at an absorption 10% higher (16.6% higher based on water content) than the normal absorption. The two Kansas flours series baked were designated as A and B series, the Nebraska series as C. In the B series the dough at the maximum absorption was too slack to mould. Weights were recorded for total ingredients, dough, and baked bread. Compressibilities were determined in the manner previously indicated at 24, 48, and 72 hours.

Table I shows that the total per cent losses of volatile materials during fermentation and baking to be constant within one range of experimental error. Accordingly, the amount of water left in the finished bread is, within the limits of experimental error, roughly proportional to the amount of water added to the dough.

The compressibilities for the three series were so similar at each bread age that they were averaged. The resulting averages, plotted in Fig. 3, show that the additional water above the farinograph absorption retarded the firming process. However, with approximately 10% extra water, the bread baked without POEMS did not show the compressibility of bread containing 0.5% POEMS but with no extra water. It appears, therefore, that the mechanism involved when POEMS retards the firming of bread is not one involving simply the plasticizing effect of water. Also, there is no evidence from Table I that POEMS has any effect on moisture losses during baking.

### Changes in Crumbliness as a Measure of Staling

Katz (10) in 1912 attempted to use crumbliness as a rough measure of staleness. He simply rubbed the crumb with his finger and noted whether the bread in question was more or less crumbly than that of a second loaf. Selman in 1943 (unpublished data) developed a mechanical method for the determination of crumbliness of cakes by the use of a standard screen in a shaking device. Bice and Geddes (3) modified the method and used it as a measure of the staleness of bread. With breads of similar composition, this method gives a fair measure of the degree of staleness.

TABLE I  
EFFECT OF EXTRA WATER ON BAKING LOSSES AND ON BREAD SCORES

| Poems (%) | Absorption* (%) | Baking Loss (%) |      |      | Score** |    |    |
|-----------|-----------------|-----------------|------|------|---------|----|----|
|           |                 | A               | B    | C    | A       | B  | C  |
| 0         | 60              | 14.9            | 13.5 | 13.1 | G—      | G  | G— |
| 0         | 62              | 16.7            | 13.2 | 12.4 | G—      | G  | G— |
| 0         | 64              | 15.0            | 13.8 | 11.6 | G—      | G  | G— |
| 0         | 66              | 15.0            | 13.0 | 12.7 | G—      | G— | F+ |
| 0         | 68              | 16.0            | 12.4 | 15.2 | F+      | F+ | F+ |
| 0         | 70              | 14.8            |      | 13.6 | F       | P  | P+ |
| 0.5       | 60              | 15.2            | 13.3 | 13.5 | G       | G  | G  |
| 0.5       | 62              | 16.9            | 15.8 | 14.5 | G       | G  | G— |
| 0.5       | 64              | 15.4            | 12.5 | 13.2 | G       | G  | G  |
| 0.5       | 66              | 17.0            | 10.9 | 13.1 | G       | G— | G— |
| 0.5       | 68              | 15.3            | 11.8 | 14.5 | G—      | G— | F  |
| 0.5       | 70              | 13.9            | 16.9 | 14.0 | G—      | F+ | P  |

\* Series C had absorption 0.5% higher than indicated by table.

\*\* Scores are average for three consecutive days. G = Good; F = Fair; P = Poor.

With the aid of a miter box, three slices, each 1 in. thick, were cut from the center portion of the loaf. Using a second miter box, four 1-in. cubes were cut from the center of each slice. The twelve 1-in. cubes were weighed, placed in a No. 4 standard sieve and shaken in a Ro-Tap machine for 30 minutes at room temperature. The "throughs" were weighed and crumbliness was calculated as the weight of crumbs obtained from 100 gm. of bread. Before determining the crumbliness on the third day the remaining wrapped loaves were heated at 200°F. (93.3°C). for twenty minutes. Two hours later, crumbliness was determined for each of the loaves.

Figure 4 shows the increase in crumbliness for loaves from five doughs containing from 0% to 2.0% POEMS. If the degree of crumbliness is taken as a measure of staleness, bread containing 2% POEMS is more stale after six hours out of the oven than the control bread after 48 hours. Reheating the bread gave, as indicated by the dotted lines in Fig. 4, slight changes in crumbliness but did not restore the original crumbliness. Three of the five loaves showed an *increase*

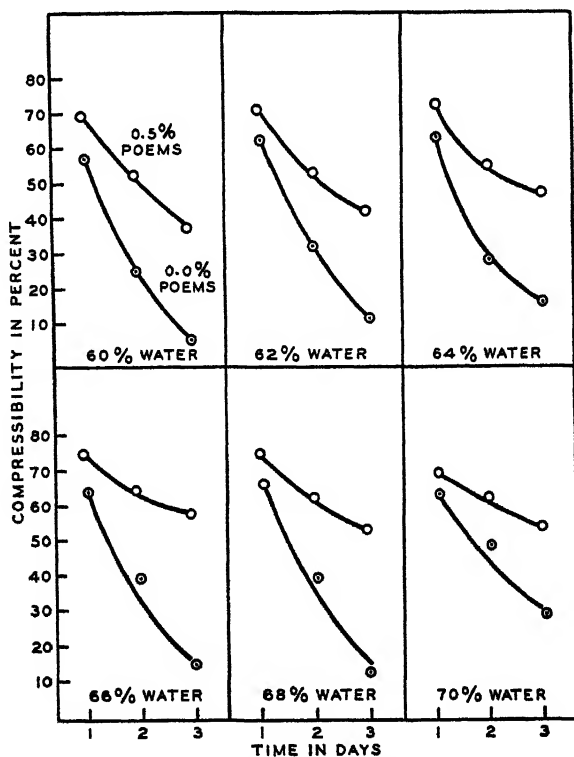


FIG. 3. Relation of water and polyoxyethylene monostearate to crumb compressibility.

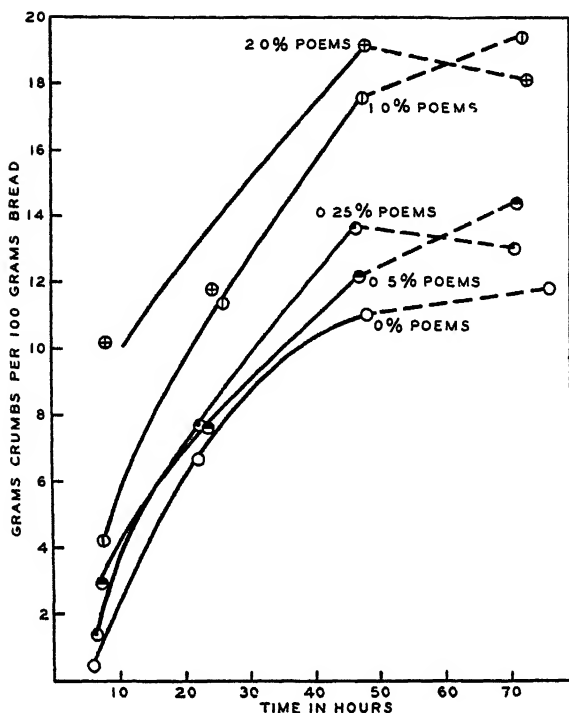


FIG. 4. Effect of polyoxyethylene monostearate on retardation of crumbliness in bread

in crumbliness. The crumbliness of the five loaves appeared to increase in approximate proportion to the content of POEMS. The results cast, at least, a reasonable doubt on the value of the test as a measure of staleness.

### Changes in Crumbliness as a Measure of Tenderness

All loaves tested in the previous section contained 2% lard. Since crumbliness did not appear to be a valid test for staleness, it was thought that it might be used as a measure of tenderness. Bread was baked by the method indicated earlier except that in one series of loaves, lard was used at 0.5%, 1.0%, 1.5%, and 2.0% levels without POEMS. In the second series POEMS was used at the corresponding levels without lard. Duplicate series were baked for determination of the relative crust tenderness by the penetrometer method described below; in these series, lard was used at levels up to 3.0% in 0.5% increments. Previous experiments had indicated that penetrometer tests were least erratic when carried out with bread approximately 44 hours old; so all loaves were doubly wrapped and heat-sealed in waxed paper and stored

TABLE II  
RELATIVE CRUST TOUGHNESS OF BREAD BAKED WITH LARD OR POEMS

| Lard<br>% | Poems<br>% | Toughness<br>Seconds to<br>Penetrate<br>Crust <sup>1</sup> |
|-----------|------------|------------------------------------------------------------|
| 0         | 0          | 38.9                                                       |
| 0         | 0.5        | 20.1                                                       |
| 0.5       | 0          | 31.4                                                       |
| 0         | 1.0        | 21.0                                                       |
| 1.0       | 0          | 29.7                                                       |
| 0         | 2.0        | 16.8                                                       |
| 2.0       | 0          | 29.9                                                       |
| 2.5       | 0          | 29.7                                                       |
| 3.0       | 0          | 27.3                                                       |

<sup>1</sup> Average of 6 determinations

at room temperature for 44 hours. Crumbliness was determined as indicated in the previous section.

For the determination of crust tenderness a very simple penetrometer was devised. A spherically tipped aluminum rod 6.25 in. long and 0.25 in. thick was attached to an aluminum funnel. A glass tube serves as a guide for the rod. An automatic shot loading device operated by an electric motor with a reduction gear was used to load the

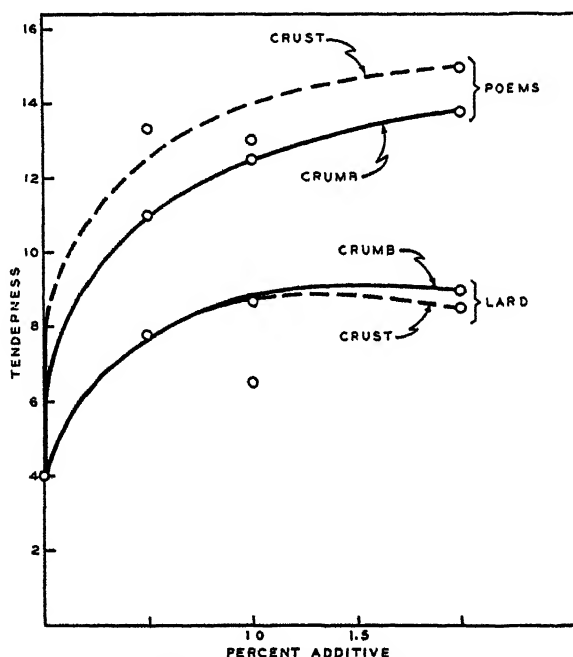


FIG. 5. Effect of hydrophobic additives on tenderness.

funnel. Since the shot is added at a constant rate, the time required for the crust to be penetrated by the aluminum rod was measured with a stop watch and the toughness of the crust recorded in seconds. A template was used to insure a uniform placement of punches, three of which were made on each side of each loaf.

The penetrometer readings correlated well with the estimates of four experienced bread judges who were asked to divide six loaves into two groups on the basis of crust tenderness. The penetrometer and the judges agreed on 22 out of 24 judgments. In a series of loaves in which the variables were lard and POEMS the relative toughness determined for each loaf, is shown in Table II.

To obtain a better conception of the relative effects of POEMS on crust toughness and crumb tenderness, the mathematical device of expressing these characteristics on a comparable scale was used. The equation:  $\text{tenderness} = (46.9 - \text{toughness})/2$  gave arbitrary values which could be plotted on the same scale.

The two sets of curves for crust and crumb tenderness as obtained above are shown in Fig. 5. Both sets of curves show that the addition of 0.25% of POEMS produces bread of approximately the same tenderness as the use of 2.0% of lard.

### Changes in Water Absorptive Capacity of Bread Crumb as a Measure of Staling

*Farinograph Method.* With the advent of the farinograph, Fuller (7) suggested that the swelling test devised by Katz (10) be modified to make use of the new instrument. Fuller preferred to titrate the dough to a definite consistency and to use the absorption thus found as an index of staling. Later Bice and Geddes (3) developed a method involving the measurement of crumb dough consistency at constant water content. Freilich (8) used their method but measured the consistency at a slightly different water content. All agreed that the farinograph offered promise as a method of measuring staling but the method did not give results comparable to those obtained by compressimetric measurements when applied to breads containing POEMS (3, 8).

Bread from hard winter wheat flour was baked by the standard procedure indicated earlier. Half the loaves contained 1% POEMS based on the weight of the flour. After being stored overnight in doubly-sealed waxed paper, the loaves were decrusted and the crumb cut into small cubes about  $\frac{1}{4}$  in. to the side. The bread was stored in air-tight containers during the testing period. Fifty-gram samples of the crumb were placed in the farinograph bowl, the motor started, and water added from a pipette until a consistency of 500 Brabender units

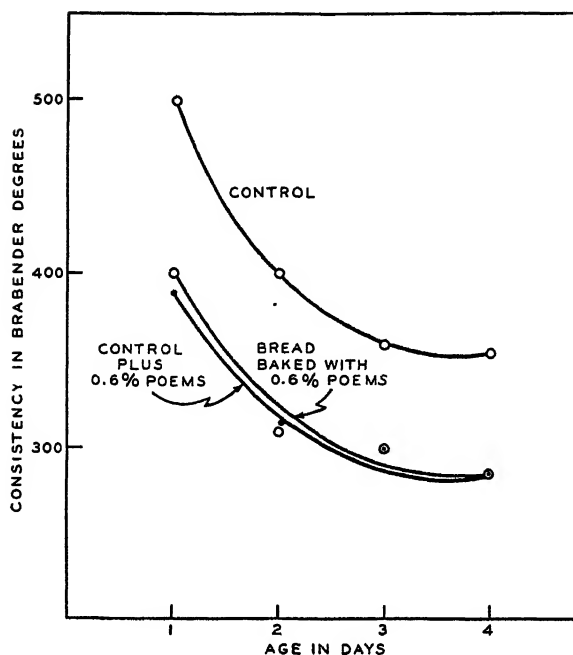


FIG. 6. The effect of age on farinograph consistencies of bread-water dough.

was obtained for the control bread. For the control bread 20.3 ml. of water were required. Three determinations were made each day, using the same quantities of crumb and 20.3 ml. of water; one determination for the control bread, one for bread containing 1% POEMS, and one for the control bread to which the same quantity of POEMS was added in the farinograph.

The results in Fig. 6 show that for bread at any age, POEMS decreased the consistency of the crumb-dough at constant water content. POEMS added in the farinograph had the same effect as POEMS baked in the bread.

TABLE III

THE RELATIONSHIP OF POLYOXYETHYLENE MONOSTEARATE TO THE STALING OF BREAD AS MEASURED BY THE SWELLING POWER TEST

| Swelling Power: Weight of Sediment per Gram of Crumb |          |            |            |            |            |
|------------------------------------------------------|----------|------------|------------|------------|------------|
| Day                                                  | 0% POEMS | .25% POEMS | 0.5% POEMS | 1.0% POEMS | 2.0% POEMS |
| 1st                                                  | 3.5      | 3.5        | 3.4        | 3.4        | 3.2        |
| 2nd                                                  | 3.5      | 3.1        | 2.9        | 2.9        | 2.9        |
| 3rd                                                  | 3.9      | 3.0        | 3.4        | 3.1        | 2.8        |
| 4th                                                  | 4.4      | 3.3        | 3.5        | 3.1        | 2.7        |

**Swelling Power Test (Katz Method).** Crumb swelling power was determined as described in Cereal Laboratory Methods, 5th Edition (5), except that the specified 25 gm. sample was reduced to 10 gm. to facilitate the subsequent determination of soluble starch in the decantate. The amount of water was proportionately reduced.

The results were most erratic (Table III). The control bread actually increased in swelling power with age while that containing POEMS remained essentially the same.

### Changes in Soluble Starch Content as a Measure of Staling

According to Katz (10), most of the changes in soluble starch take place during the first eight to ten hours after the bread is removed from the oven. From a practical viewpoint this reduces the test to one of academic interest only, since no housewife receives commercial bread less than ten hours old.

TABLE IV  
THE RELATIONSHIP OF POLYOXYETHYLENE MONOSTEARATE TO THE  
STALING OF BREAD AS DETERMINED BY SOLUBLE STARCH

| Day | Per Cent Soluble Starch |             |            |            |            |
|-----|-------------------------|-------------|------------|------------|------------|
|     | 0% POEMS                | 0.25% POEMS | 0.5% POEMS | 1.0% POEMS | 2.0% POEMS |
| 1st | 4.5                     | 4.9         | 4.2        | 4.3        | 4.6        |
| 2nd | 4.2                     | 4.0         | 4.1        | 4.2        | 4.1        |
| 3rd | 3.4                     | 4.1         | 3.9        | 3.3        | 4.1        |
| 4th | 3.6                     | 4.0         | 4.0        | 3.7        | 3.8        |

Bread was baked containing 0%, 0.25%, 0.5%, 1.0%, and 2.0% POEMS and soluble starch in the crumb determined at one-day intervals for four days by the method of Katz (10). The data are recorded in Table IV and are so erratic that no conclusions can be drawn regarding the effects of POEMS on the rate of staling.

### Effect of POEMS on Wetting Rates of Bread Crumb

Loaves were baked containing 0%, 0.5%, 5.0% POEMS and 1.0% sodium lauryl sulfate based on the weight of flour. The sodium lauryl sulfate used was desalted before being incorporated into the dough. The breads were air-dried and ground under the same conditions to pass a 20-mesh screen.

The wetting-rate test was a modification of the one used for textiles. A weighed sample (usually 5 gm. except as otherwise indicated in Table V) of the ground bread was poured through a wide-mouthed funnel onto the surface of 450 ml. of distilled water in a 500-ml. graduated cylinder, and the time required for the material to sink was



TABLE V  
RELATIVE WETTING TIMES OF DRY GROUND BREAD WITH  
AND WITHOUT ADJUNCTS

| Adjunct                  | Seconds for Sample To Sink |      |        |       |      | Average |
|--------------------------|----------------------------|------|--------|-------|------|---------|
|                          | 1                          | 2    | 3      | 4     | 5    |         |
| None                     | 185                        | 171  | 140.2* | 176.8 |      | 177.6   |
| 5.0% POEMS               | 214                        | 205  | 195    | 225   |      | 210     |
| None**                   | 201.2                      |      |        |       |      | 201.2   |
| 5.0% POEMS**             | 696.8                      |      |        |       |      | 696.8   |
| 0.5% sod. lauryl sulfate | 55.0                       | 62.0 | 72.8   | 51.8  | 45.2 | 51.2    |
| 1.0% sod. lauryl sulfate | 30.2                       | 40.8 | 55.4   | 32.2  | 49.6 | 41.6    |

\* Not included in average.

\*\* 10-gram samples.

measured with a stop watch. The instant at which the last particle of bread lost its opaque appearance was taken as the endpoint.

Table V shows that the wetting time of bread was inversely proportional to the amount of sodium lauryl sulfate added, but directly proportional to the amount of POEMS present. Bread containing no adjunct took longer to wet than bread containing sodium lauryl sulfate, but bread containing POEMS took longer to wet than the control bread. The results indicate that, unlike sodium lauryl sulfate which makes bread more hydrophilic and speeds up the wetting rate, POEMS makes bread less hydrophilic and retards the wetting rate.

### Discussion

It appears that POEMS does not prevent staling; it merely retards the process. This retardation of staling may be caused by POEMS precipitating amylose which is present in a free condition in the bread. But combined with the precipitation, and probably of much more importance, is the action of POEMS on the starch granules themselves. POEMS appears to combine, probably by hydrogen bonding with alcoholic groups protruding from the starch granule, with the granules themselves. This addition of a hydrophobic moiety hinders to some extent the forces which cause alignment and hydrogen bonding of starch chains with each other and the subsequent hardness which develops when bread stales. This interference retards the staling process but does not stop it.

If this mechanism of the action of starch is acceptable, and no other mechanism appears at this time to explain the laboratory findings, it appears reasonable that those staling tests which depend on the action of water at or near room temperature cannot be expected to give normal indications. Of all the traditional tests for staling, only those involv-

ing the measurement of compressibility and crumbliness appear to give results which can be interpreted as indicating the degree of bread staling, and crumbliness appears to be a better measure of tenderness than of bread age.

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## AN AUTOMATIC ELECTRICAL RECORDING PRESSUREMETER <sup>1</sup>

SUTTON REDFERN <sup>2</sup>

The pressure of the gas produced during fermentation in a sealed vessel is automatically recorded by a 12 point strip chart Brown Electronik potentiometer. Pressure is measured with a Satham Pressure Transmitter which translates pressure into a voltage linearly proportional to the pressure. The potentiometer is calibrated directly in millimeters of mercury and can record a total pressure range of 0-600 mm. Twelve separate pressures may be recorded simultaneously. The various components of the apparatus, recorder, pressure transmitters, power supply, pressure vessels, pulley box, and water bath are described.

Many papers, reviewed by Elion (6), have been published dealing with the measurement of gas production in fermenting dough. Two

<sup>1</sup> Manuscript received April 26, 1950. Presented at the annual meeting, May, 1948.

<sup>2</sup> The Fleischmann Laboratories, Standard Brands Inc., New York, N. Y.

methods are generally used to measure the amount of gas produced: the volumetric method as described by Bailey and Johnson (1) and the manometric method originated by Blish, Sandstedt, and Astleford (3), from whose work was developed the well known pressuremeter of Sandstedt and Blish (14) which is so widely used today. Both of these methods are usually manually operated, i.e., the operator takes readings every hour or even oftener. Few automatic recording types of equipment have been constructed, and of those that have been offered commercially only a very limited number are in use in this country.

Markley and Bailey (12) described a recording fermentometer in which air displaced by the expanding dough displaces water in a bell jar. This displaced water is discharged into a cylinder equipped with a float and stylus. The height of the stylus, which is proportional to the volume of displaced air, is recorded on a conventional kymograph chart.

Working and Swanson (17) constructed a simple recording pressuremeter in which the pressure of the gas produced during fermentation actuates a stylus floating in a mercury manometer. The height of the stylus, which is proportional to the pressure, is automatically recorded by electric sparks which burn holes in a chart fastened to a kymograph drum.

At least three devices for recording gas production have been offered commercially. These are the fermentograph of Brabender (2), the Chefaro balance described by Elion (5), and the S.I.A. Fermentation Recorder (9). In the Fermentograph a rubber balloon containing the fermenting dough is suspended from a recording balance in water. As the balloon expands it becomes more buoyant and the rise is recorded by the balance. Although very attractive in principle, Schmalz and Sullivan (15) showed that the error due to diffusion of carbon dioxide through the rubber balloon was too large to be neglected. The Chefaro balance is similar in principle to the Fermentograph but places the dough under an open bell jar. The S.I.A. apparatus, which is similar to the Benedict-Roth (7) spirometer, measures the volume of fermentation gas with a floating gasometer bell. As far as is known, the Fermentograph is the only one of these instruments available today.

The instruments so far mentioned can be classed as integrating instruments in that the total amount of gas is recorded. For some purposes the instantaneous rate of gas production is very important. A number of investigators (4, 16) have determined average rates by measuring the increment of gas produced during successive intervals of time where this interval has been as small as five minutes. These

data can of course be obtained from any of the integrating types of instruments.

Miller, Edgar, and Whiteside (13) constructed an instrument which automatically recorded the pressure produced during every successive ten minute interval. Landis and Frey (10) used a sensitive optical type differential recording manometer to measure the instantaneous rate of fermentation. Earlier, James and Huber (8) made similar instantaneous measurements by allowing the water displaced from a tank by the fermentation gas to flow through an orifice under gravity. The height of water above the orifice was proportional, although not linearly, to the rate of gas production and the height was recorded by a stylus floating in the water.

The development during recent years of improved recording potentiometers, electrical pressure transmitters, and power supplies have made it possible to construct the automatic electrical recording pressuremeter described in this paper. This recording pressuremeter can be assembled from readily available standardized components. Such an instrument should make it possible to examine more exactly the entire course of yeast fermentation in dough.

### Materials and Methods

*General.* A general view of all components except the power supply is shown in Fig. 1. The pressure of the gas produced during fermentation by a piece of dough in special sealed cups is automatically recorded by the 12-point strip chart Brown Electronik potentiometer shown on the left. The potentiometer is calibrated directly in millimeters of mercury and can record a total pressure range of 0 to 600 mm. Pres-

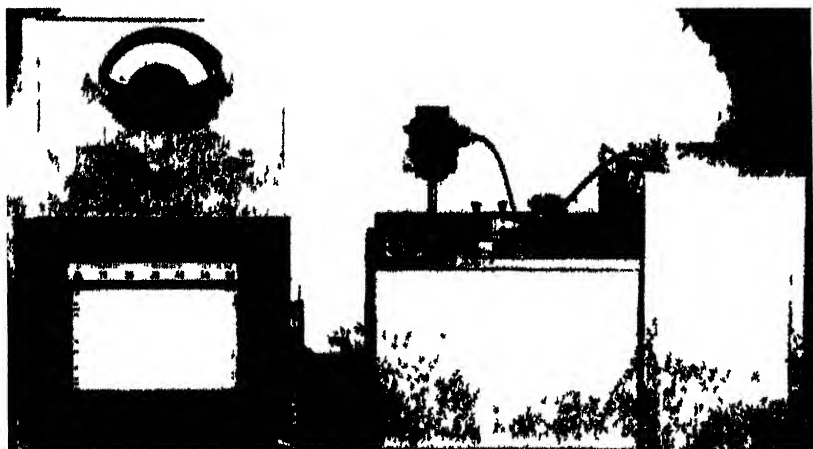


FIG. 1 General view of recording pressuremeter

sure is measured with a Statham pressure transmitter which converts pressure into a voltage linearly proportional to the pressure. A pressure transmitter mounted on its cup in place in the water bath is shown in the center of Fig. 1. On the right is the so-called pulley box. This contains the necessary wiring for connecting each pressure transmitter to the potentiometer and to the power supply and the zero adjustment and calibrating resistors. The pulley box is connected to the potentiometer with a 30-wire cable. The voltmeter mounted over the potentiometer indicates the voltage of the power supply.

*Statham Pressure Transmitter.* The heart of the pressuremeter is the Statham pressure transmitter shown mounted on a pressure cup in Fig. 2. The pressure to be measured is applied through the bottom opening to a relatively slack metal bellows and the resulting displacement of the bellows is measured with a Statham unbonded resistance wire strain gage transducer element. Of the various models of pressure transmitters available, Model P6-12D-250 was chosen as most suitable. This transmitter is designed to measure 12 lb. maximum pressure and has a nominal electrical resistance of 250  $\Omega$ . The output is approximately 36 mv. with an input voltage of 12 v.

*Pressure Cup.* Special pressure cups,  $2\frac{1}{2}'' \times 4\frac{7}{16}''$ , approximately 300 cc. in volume, were constructed of stainless steel. This cup, shown in Fig. 2, is patterned after the small De Vilbiss paint spray cup used by Malloch (12). The cup body is made from  $2\frac{1}{2}''$ , 16 gauge (approximately  $1/16''$ ), stainless steel sanitary tubing. This tubing comes already polished so that it needs no further finishing. The bottom and the ring at the top were induction soldered to the tubing with silver solder. The extra ring at the top was added to give increased gasketing surface. The cover is turned from solid bar stock in the form of a frustrum of a cone. A  $3/32''$  thick soft rubber gasket is cemented in the annular space on the bottom of the cover. The screw is made from  $1\frac{1}{8}''$  hexagon stock. The stem, turned from 1'' round bar stock, has a  $1/8''$  tapered pipe thread at the top for connection to the pressure transmitter and a  $7/16-20$  thread at the bottom which is soft soldered to the cover. In the future it is planned to eliminate soldering by using  $1/8''$  pipe threads for both threads. The yoke is specially cast from phosphor bronze. The release valve is a Hoke blunt point baby valve.

This pressure cup design has proved very satisfactory. No trouble from leaks or corrosion has occurred, and the cover can be easily tightened without using a special holding stand and wrench. The entire assembly may be tested for leaks by attaching a hose connection to the valve, adding 10 to 12 lb. air pressure, and immersing the whole in water. The most minute leak can be readily detected.

*Recording Potentiometer.* The output from each pressure transmitter is recorded on a Brown Electronik strip chart potentiometer (series 153 X 65, fast speed, synchro-printing, 12 record). This model prints 12 multi-colored records by means of a print wheel. Each record consists of one of six different colored numbers and plus signs, each plus sign (+) representing the exact pressure and time coordinate. Chart speeds of 1, 2, 3, and 4 inches per hour are obtainable with the change

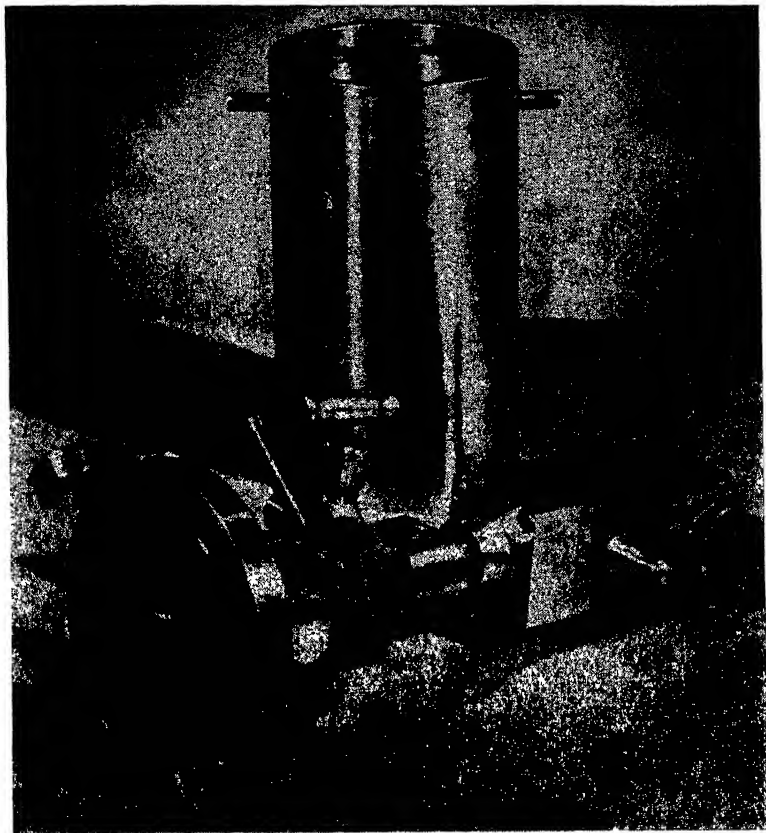


FIG. 2. Pressure cup and Satham Pressure Transmitter.

gears furnished, although 4" per hour is used for fermentation times lasting 4 to 6 hours. The scale has a calibrated length of 11" and is evenly graduated 0 to 60 equal to 0 to 600 mm. of mercury pressure.

The actual electrical span is 25 mv., which was deliberately chosen smaller than the maximum output available from the pressure transmitters. This makes it possible to use transmitters of varying sensitivity as it is very difficult for the manufacturer to exactly predeter-

mine the output of individual pressure transmitters. Matching of the transmitter output to the recorder is described under the heading of calibration.

It was found necessary to connect a one microfarad paper capacitor from the positive terminal in the recorder to ground in order to by-pass a stray alternating current component picked up by the pressure transmitters or their connecting wires from the Nobatron power supply.

The recording potentiometer contains an integral commutator switch which connects each pressure transmitter in sequence. The connections from the switch terminal block in the instrument are all brought to terminal strips placed below the voltmeter, and which in turn are connected to a Type K, 34-wire Cannon connector mounted on the back of the potentiometer mounting frame.

*Power Supply.* A Model E-12-5 Nobatron made by the Sorensen Company, is used to supply constant voltage direct current for the input to each pressure transmitter. The Nobatron which is electronically controlled will supply 5 amp. at 12 v. with a variation in output voltage of less than 0.5%, even when the line voltage varies from 95 to 125 v., and the load varies from 2.5 to 5 amp. Since 12 pressure transmitters will require not more than 1 amp., a 5  $\Omega$ , 50 w., bleeder resistor is shunted permanently across the output of the Nobatron to insure a minimum load of 2.5 amp. and bring the load within the range of best regulation. The output voltage may be varied over a limited range of  $12 \pm 10\%$  v. but is maintained constant at 12 v. as measured by the voltmeter mounted over the potentiometer (Fig. 1).

The Nobatron was mounted as far away from the pressure transmitters as possible in order to minimize pickup of stray currents from the strong magnetic fields surrounding it.

*Pulley Box.* All wiring and controls for operation of the pressure transmitters are consolidated in the pulley box shown on the right in Fig. 1. The wiring although simple in principle is complex in that there is a lot of it. Moreover, some ready means must be used to quickly connect the pressure transmitters at varying distances from the pulley box without permitting the connectors to fall into the water bath. This entire problem was solved by modeling the pulley box after a telephone switchboard.

The wiring connections necessary for each pressure transmitter are shown diagrammatically in Fig. 3. Terminals 1, 2, 3, and 4, are numbered to correspond to the terminals of the Wheatstone bridge circuit in the pressure transmitter. Terminals 2 and 3 are connected permanently to terminal strips. Terminals 4 and 5 are connected to bus bars which in turn are connected respectively to the positive and negative sides of the power supply. Resistor  $R_3$  is a 200  $\Omega$  wire wound





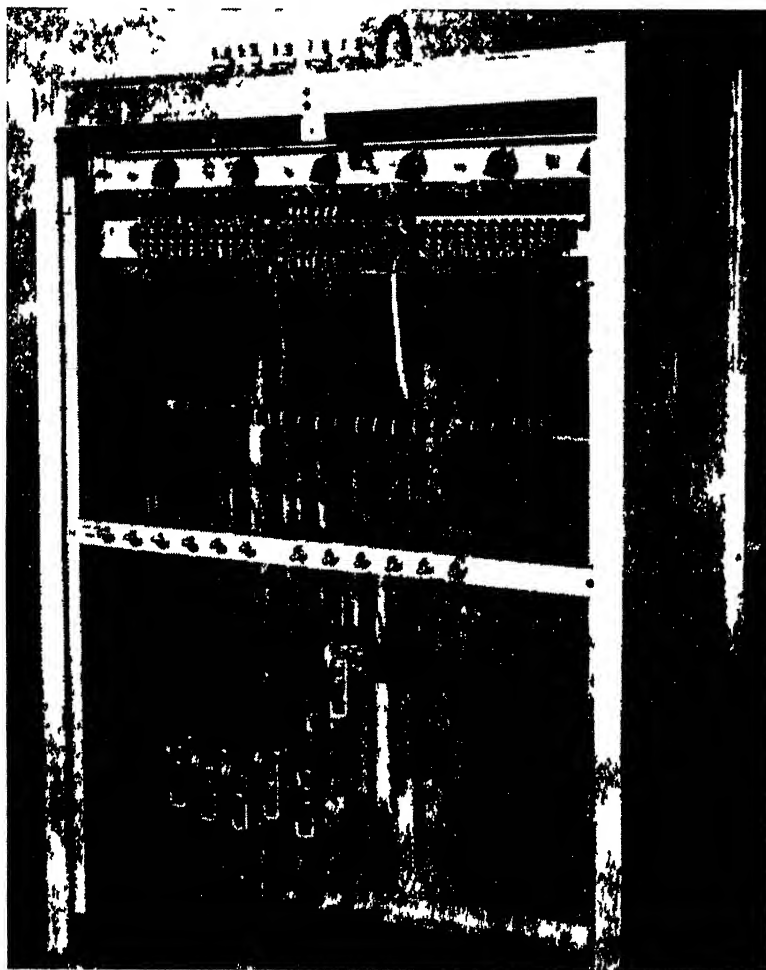


FIG. 4 Interior view of pulley box.

bath as the weighted pulley draws it back into the box whenever it is released.

The terminal strips, bus bars, zero adjustment, calibrating resistors, and shorting switches are readily identified in Fig. 4. The shaft of the calibrating resistor is slotted which allows it to be adjusted only with a screwdriver which prevents it from being accidentally turned.

The entire wiring arrangement proved to be very flexible since a number of variations and additions were made directly to the terminal strips without disturbing existing wires. It is planned eventually to mount each shorting switch conveniently adjacent to its respective Cannon connector on the top of the pulley box.

*Water Bath.* The pressuremeters are immersed in a conventional thermostatically controlled water bath (Fig. 1) containing a 12 place rack. The holes in the rack are diagonally arranged so that the wires to the pulley box are separated.

*Calibration of Pressure Transmitters.* Although the pressure transmitters are factory calibrated, the output of different transmitters cannot be made to give exactly the same output voltage for the same pressure. The input voltage must be adjusted until the pressure indicated on the potentiometer equals the pressure measured with a mercury manometer. This is done by attaching each pressure transmitter and cover to the calibration unit shown in Fig. 5 made from a Sandstedt-



FIG. 5. Apparatus used for calibrating pressure transmitters.

Blish manometer pressuremeter. Air pressure is applied through the Hoke valve and the calibrating resistor adjusted until the pressure read on the potentiometer equals the pressure read on the manometer. A calibrating pressure nearly equal to full scale is used and the calibration is repeated until one is certain that the calibration is correct.

*Operation of Pressuremeter.* The sequence of operations in the use of the pressuremeter is as follows: The Nobatron is turned on 10 to 15 minutes before use in order to let it warm up and develop a stable output and the output is adjusted to 12 v. The Brown Electronik potentiometer should be operated according to the directions of the manufacturer. The power supply is best left on continuously since this procedure is stated to give longer tube life. The wiring of the potentiometer should be fixed so that it will continuously indicate even when the chart drive switch is turned off.

After each pressure transmitter is connected to the pulley box, it requires about a five minute warm-up period before the output becomes steady. This is particularly noticed with the zero setting and one should not be in haste to make any changes in the zero adjustment until the warm-up period is over. When five minutes or more has elapsed, the output with no pressure applied to the transmitter is set to zero with the adjusting resistor. There are slight daily zero shifts of about  $\pm 2$  mm., and for the highest accuracy the zero should be adjusted each day. The amount that the resistor must be moved is readily correlated with the amount of zero shift.

To measure fermentation pressure, a suitable sized dough aliquot, which may vary from 25 to 50 gm. is placed in the cup, or alternately the dough may be mixed in the cup and the cover affixed tightly with the valve open. The cup is then immersed in the water bath, the pressure transmitter connected to its corresponding Cannon connector, and the shorting switch opened. After at least five minutes have elapsed, the zero point is adjusted and the valve closed exactly ten minutes from start of mixing.

The potentiometer chart is positioned so that the first point prints on one of the principal time lines. Successive doughs can be started as close as five minute intervals.

## Results

*Relation of Response to Pressure.* According to the manufacturer, the pressure transmitters exhibit a linear relationship between applied pressure and electrical output with an error of less than one per cent of full range from hysteresis and creep. The linearity of response was tested by attaching one of the pressure transmitters to the calibration unit and comparing pressures measured on the manometer with the

TABLE I  
RESPONSE OF PRESSURE TRANSMITTER

| <i>Potentiometer<br/>mm.</i> | <i>Pressure</i> | <i>Manometer<br/>mm.</i> |
|------------------------------|-----------------|--------------------------|
| 0                            |                 | 0.0                      |
| 51                           |                 | 51.5                     |
| 100                          |                 | 101.0                    |
| 150                          |                 | 151.0                    |
| 200                          |                 | 201.5                    |
| 250                          |                 | 251.5                    |
| 300                          |                 | 301.0                    |
| 350                          |                 | 351.5                    |
| 400                          |                 | 400.0                    |
| 450                          |                 | 449.0                    |
| 500                          |                 | 499.0                    |
| 550                          |                 | 548.0                    |
| 599                          |                 | 597.0                    |

pressures indicated on the potentiometer. The data, shown in Table I deviate only slightly from a linear relationship. In general, the performance of the pressure transmitters has been better than the stated 1%, probably because they are used essentially for measurement of static pressures. The pressure changes are relatively slow and each transducer element is essentially always in a state of equilibrium, which reduces any hysteresis or creep effects.

The pressuremeter may be used for any method for which the manually operated pressuremeters have been used. Typical curves obtained for doughs containing 3% yeast, no salt, and 0, 1, 2, 3, and 5% sugar are shown in Fig. 6. The transference from sucrose ferment-

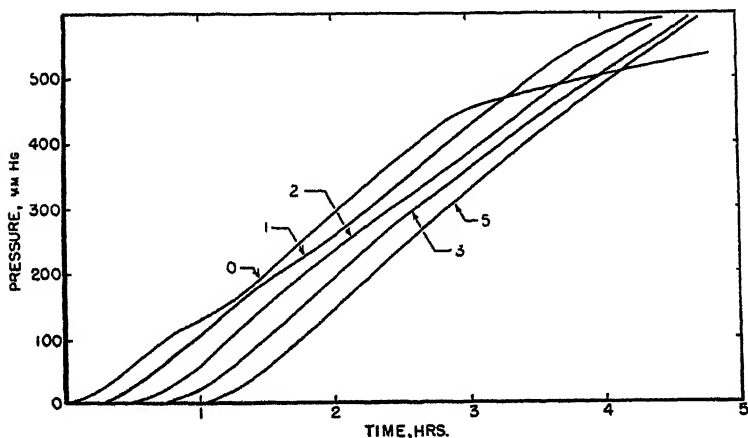


FIG. 6. Typical pressuremeter curves obtained for doughs containing 3% yeast, no salt, and 0, 1, 2, 3, and 5% sucrose. (In the chart actually obtained from the recorder, the time axis runs from right to left.)

tation to maltose fermentation is clearly shown by the inflection points in the curves from doughs containing none, 1 and 2% sugar. With 5% sugar, the inflection point has disappeared. Differentiating the pressure time curves would show this condition more emphatically, but it is believed that these are the first curves which show this transition so clearly.

Some idea of the replicability of the recording pressuremeter is shown by Table II. These are the hourly rates and total pressure of six aliquots of the same dough. Zero time is taken as  $\frac{3}{4}$  hour after mixing. The reason for choosing this starting time is because although all the cups were placed in the water baths simultaneously, recording was started at successive five minute intervals in order to separate the curves. The initial portion of most of the curves is thereby missed.

TABLE II  
REPLICABILITY OF PRESSUREMETER READINGS  
(Six 25 gm. aliquots from same dough)

| <i>Hour</i> | <i>Pressure</i>      |                        |
|-------------|----------------------|------------------------|
|             | <i>Range<br/>mm.</i> | <i>Average<br/>mm.</i> |
| 1           | 86-88                | 87                     |
| 2           | 115-117              | 116                    |
| 3           | 118-120              | 119                    |
| 4           | 108-110              | 109                    |
| 5           | 99-100               | 100                    |
| Total       | 527-532              | 530                    |

This new recording pressuremeter has not been in operation long enough to have explored its many uses and possibilities. It may eventually be used for directly recording actual fermentation rates with a suitable pressure transmitter.

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## WET MILLING OF GRAIN SORGHUM<sup>1,2</sup>

R. L. ZIPF, R. A. ANDERSON, and R. L. SLOTTER<sup>3</sup>

### ABSTRACT

Studies on the recovery of starch, gluten, and oil from grain sorghum showed that this grain could be processed satisfactorily by methods similar to those employed industrially for the wet milling of corn. Optimum separations of starch and gluten were obtained when grain sorghum was steeped at 110° to 120°F. for 24 hours or longer, in a solution of 0.2 to 0.25% sulfur dioxide in water. To produce sorghum starch of comparatively low protein content (0.35 to 0.7%) it was necessary to increase the pitch of the starch table over that used for wet milling corn.

Some varieties of grain sorghums have a highly pigmented layer. When this layer was removed by pearling, a whiter starch could be produced than from the whole sorghum grain. The hull fraction contained a carnauba-like wax, small amounts of which were recovered by solvent extraction with hexane.

Samples of grain sorghums of two varieties that had been harvested at three moisture levels and dried with air at temperatures of 125°, 150°, 175°, and 200°F. were wet milled to determine the effect of such artificial drying on the milling characteristics of the grain. It was concluded that no damage detrimental to wet-milling operations occurs when grain sorghum is artificially dried, provided the grain is not dried to less than 11 to 13% moisture.

The grain sorghums are important economically to the semi-arid regions of this country since they will thrive and produce both grain and forage under conditions that cause other crops to fail. In recent years the production of sorghum grains has increased tremendously

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<sup>2</sup> Contribution of Northern Regional Research Laboratory, Peoria, Illinois. One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

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TABLE I  
CONSTITUENTS OF CORN AND GRAIN SORGHUM

|                | <i>Corn</i> | <i>Sorghum</i> |
|----------------|-------------|----------------|
| Starch % d b.  | 70.6        | 69.5           |
| Protein % d.b. | 10.2        | 13.0           |
| Oil % d.b.     | 4.3         | 3.6            |
| Ash % d.b.     | 1.6         | 1.9            |
| Germ % d.b.    | 11.1        | 10.5           |
| Bran % d b.    | 6.5         | 6.3            |

and industrial utilization of the crop has been encouraged by the development of varieties which can be harvested with combines. Before 1943 (5) almost all of the sorghum grain and forage produced was consumed on the farm. Only a small quantity of the grain was used for food or in industry. During the war, the shortage of corn forced producers of alcohol and starch to utilize grain sorghums. It was found that the grain could be processed much like corn. Since the end of war, a large plant has been erected in Texas to wet mill grain sorghums for the production of starch, glucose syrup, and dextrose (6).

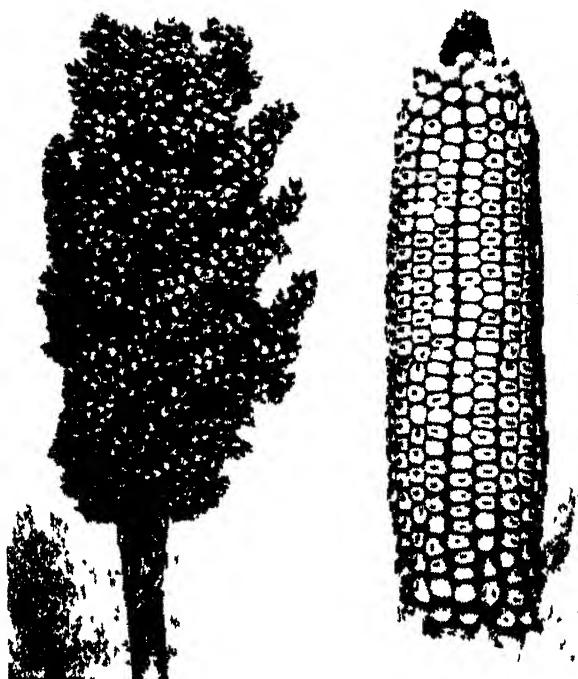


FIG. 1. Grain bearing structures of corn and grain sorghums.

Grain sorghum is a comparatively new industrial crop and a standard procedure for wet milling the grain has not been established. The industrial facilities for the wet milling of corn are extensive, however, and considerable information on the methods employed has been accumulated (2). Hence, it was logical that a procedure for wet milling grain sorghum be an adaptation of that used for corn. As shown in Table I, grain sorghum is similar in composition to corn, although in appearance it is quite different (Fig. 1). The present study was conducted to determine the suitability of the standard wet milling process for use with grain sorghum, and to ascertain the effect of variations in processing conditions on the quality and yield of products obtained from grain sorghum.

A subsequent investigation was conducted in which samples of artificially dried grain sorghums were wet milled to determine the effect of such drying on the milling characteristics of the grains. The work was conducted on samples of dried grains prepared and supplied by the Corn Products Refining Company, and the Texas Agricultural Experiment Station, College City, Texas. The results obtained by both the Texas group and the Northern Laboratory have been published (10) but a brief account of the wet-milling experiments is included in this report.

### Procedure

The general process utilized in the laboratory for the wet milling of grain sorghums is shown in Fig. 2. The procedure is approximately the same as the industrial process for the wet milling of corn, and with modifications it can be used to wet mill wheat (9).

A 4-lb. sample of grain is steeped with 2,800 ml. of distilled water containing sulfur dioxide. The operation is conducted in the equipment shown in Fig. 3. Grain is charged into the insulated bottle and covered with the dilute sulfurous acid which is prepared by bubbling gaseous sulfur dioxide into distilled water. The steeping agent is pumped continuously from the bottle through a screened opening to an oil bath from which it is returned to the bottle. The oil bath is equipped with a 1,500 watt heater and thermostat, and serves to maintain the circulating liquor at a constant temperature during steeping. Industrial steeping of corn is conducted in a countercurrent multiple contact system for about 42 hours. An important difference between the two methods is that little if any lactic acid is formed during the laboratory steeping. However, laboratory experiments with corn have shown that starch yields comparable to those of industry are obtained by steeping the grain batchwise for 24 hours.

After the grain is steeped, the water is drained off, measured, and analyzed. The steeped grain is ground in a Quaker City drug mill,



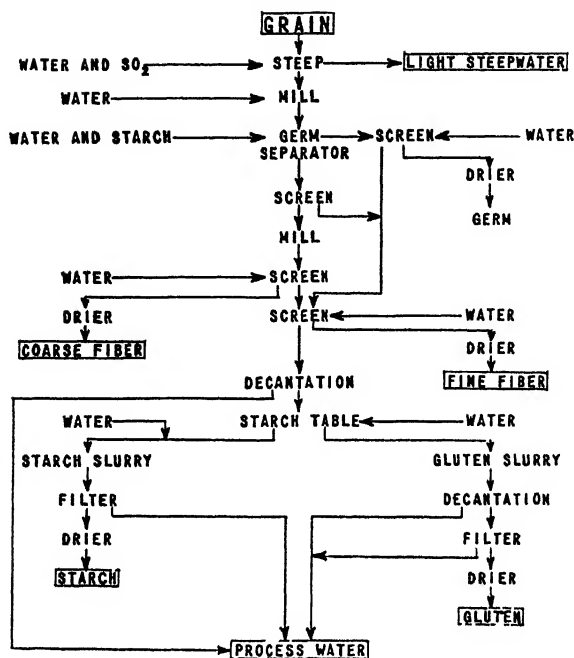


FIG. 2. Flowsheet for the wet milling of grain.

Fig. 4, to free the germ and hull from the rest of the kernel. To facilitate floating the germs from the 4 lb. of ground grain, 200 gm. of starch is added to the slurry, the specific gravity of which is adjusted with additional water to 7 to 7.5° Bé. at a temperature of 80° F. The germ material, containing a small proportion of crushed germs, is washed by hand on a 0.039-in. perforated copper screen measuring about 14 in.

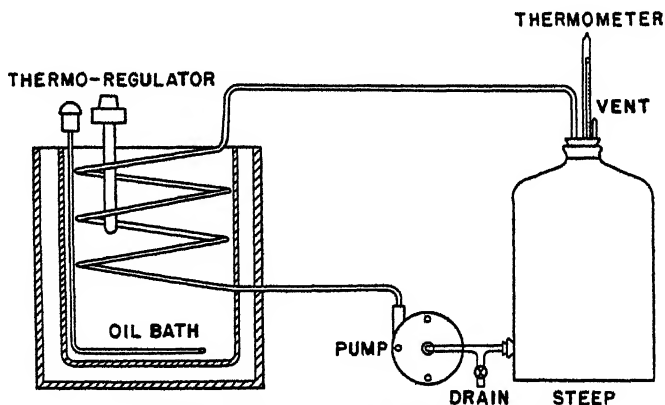


FIG. 3. Laboratory equipment for the steeping of grain sorghum.

square. The germs and 1 l. of water are mixed before each of three washes.

The slurry from which the germ has been removed is screened and the material retained on the sieve is ground again with a closer setting on the mill in order to free more starch from gritty or unbroken particles. Coarse fiber is removed from the ground mass by screening it through a 0.039-in. perforated copper sieve. The coarse fibers are washed with three 1-l. portions of water. The screened slurry is combined with the germ washings and fiber washings and the resultant liquor is screened on a 200-mesh stainless steel sieve, to remove the

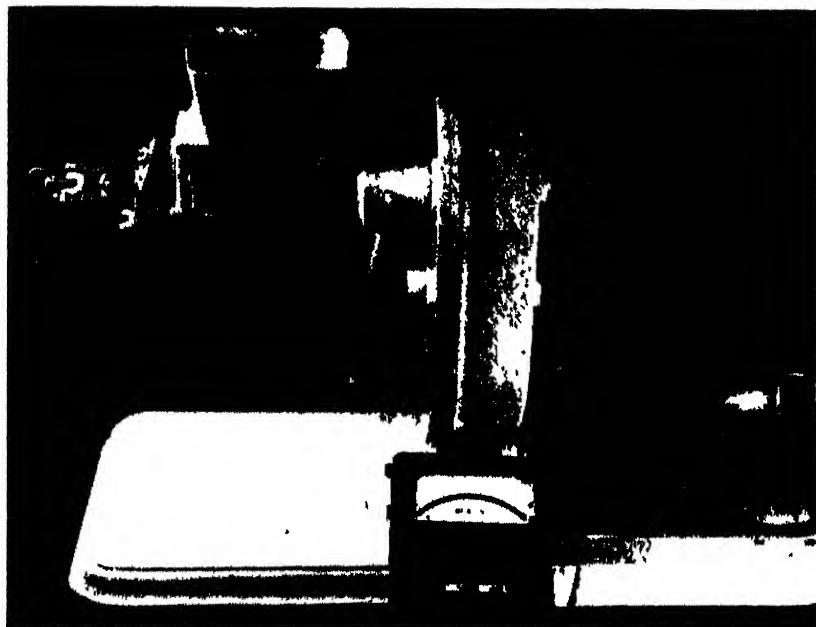


FIG. 4. Mill in which steeped grain is ground.

fine fiber, which is then washed. The final slurry containing the starch and gluten is settled for about 2 hours and excess water is decanted. The specific gravity of the liquor is adjusted to 6° Bé. at 100°F. before it is passed to the starch table. The head of the table, the mill-starch tank with accessories, and a constant-head device are shown in Fig. 5. This table, the pitch of which may be varied, is 4 in. wide and 20 ft. long. The starch that settles on the table is resuspended in water and filtered, using a Buechner funnel. The gluten is recovered from the table overflow by allowing it to settle overnight, syphoning off the supernatant water, and then filtering the settled gluten. All decanted

water and filtrates are combined, measured, and analyzed. The germs, coarse fiber, fine fiber, starch, and gluten are put on separate stainless steel trays and dried overnight at a temperature of 120°F. in a Proctor and Schwartz shelf oven.

*Analytical Methods.* Kjeldahl nitrogen in the various products was determined by the Kjeldahl-Gunning-Arnold method (1). Protein was calculated by means of the formula  $N \times 6.25$ . Starch was determined polarimetrically by the procedure of Earle and Milner (4). The

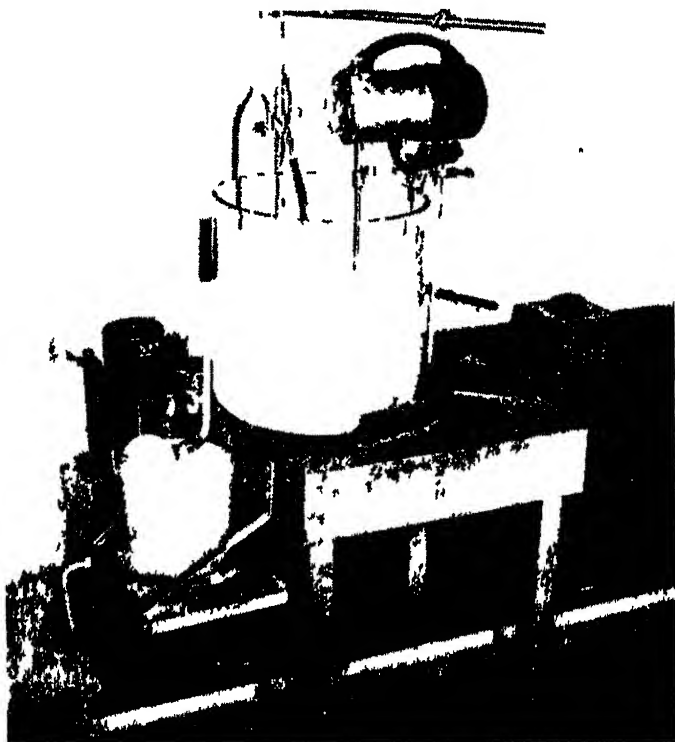


FIG 5 Laboratory starch table and appurtenances

viscosity of starch was measured according to the Scott method for hot paste viscosity (7) which is a special test used by the wet millers to evaluate their product. To determine moisture, samples were dried for 4 hours at 110°C. under a vacuum of 28 in. of mercury. The sulfur dioxide content of the steep water was ascertained by titration with a potassium iodide-iodine solution using starch as an indicator. In this paper concentration of sulfur dioxide is reported as per cent, equivalent to grams of gas per 100 ml. of solution.

## Experiments and Results

*The Effect of Concentration of Sulfur Dioxide in Steepwater.* In this and subsequent experiments the same sample of Martin Variety of grain sorghum was used unless otherwise noted. Several experiments were conducted to determine the effect of concentration of sulfur dioxide in steepwater on the wet milling of grain sorghum. It is known that excessive concentrations of sulfur dioxide have a detrimental effect on cornstarch (3), and cause drastic changes in root starches. Insufficient sulfur dioxide in steepwater usually results in lower starch yields. Only the sulfur dioxide content of the steepwater was varied in these experiments. In all runs the grain was steeped for 65 hours at 118°F. The data procured in these tests are given in Table II.

When the steepwater contained less than 0.20% sulfur dioxide, poor germ separation was obtained. Very few germs floated and the fiber content of the germ fraction was high. Germ separation was satisfactory with 0.20% and 0.25% sulfur dioxide in the steepwater.

TABLE II  
EFFECT OF CONCENTRATION OF SULFUR DIOXIDE IN STEEPWATER  
ON WET MILLING OF GRAIN SORGHUM

| SO <sub>2</sub> in Steepwater <sup>1</sup> | Starch Recovered, d.b. | Protein in Starch, d.b. | Protein in Gluten, d.b. | Starch in Gluten, d.b. | Protein in Steepwater Solids | Viscosity of Starch, Scott |
|--------------------------------------------|------------------------|-------------------------|-------------------------|------------------------|------------------------------|----------------------------|
| %                                          | %                      | %                       | %                       | %                      | %                            | sec.                       |
| 0.10                                       | 74.2                   | 0.89                    | 39.4                    | 45.3                   | 1.61                         | 127                        |
| 0.15                                       | 71.4                   | 0.73                    | 40.3                    | 46.2                   | 1.64                         | 117                        |
| 0.20                                       | 74.1                   | 0.98                    | 37.9                    | 53.0                   | 1.77                         | 113                        |
| 0.25                                       | 75.5                   | 0.62                    | 40.5                    | 47.9                   | 1.80                         | 118                        |

<sup>1</sup> % = gm./100 ml.

Varying the sulfur dioxide content of steepwater between 0.10 and 0.25% had no significant effect on the starch recovery, the protein content of the recovered starch, protein and starch contents of the gluten, or the viscosity of the starch. A slight increase in the protein content of the steepwater was obtained at the higher concentrations of sulfur dioxide. Since no detrimental effects were caused by 0.25% sulfur dioxide, all subsequent work was conducted with steepwater containing this quantity of the gas.

*Temperature of Steeping.* All samples of grain were wet milled in the regular manner but steeping temperatures of 110°, 120°, 130°, 140°, and 150°F. were used. In all cases the grain was steeped for 65 hours with water which contained 0.25% sulfur dioxide. The results of the experiments are given in Table III.

The temperature of steeping had an outstanding effect on the tabling operation. As the temperature was increased, the starch recovery, the viscosity of the starch, and the protein content of the gluten were reduced, and the starch content of the gluten increased. The quality of both the starch and gluten were impaired drastically at the high steeping temperatures. However, microscopic examination of the starch revealed that gelatinization as indicated by loss of birefringence of the granules, had not occurred at any of the temperatures. This was as expected since sorghum starch begins to gelatinize at about 158°F.

Since the starch was not damaged appreciably when the grain was steeped at 120°F., it was concluded that grain sorghum should be steeped at a temperature of 110°–120°F. with water containing 0.25% sulfur dioxide.

TABLE III  
EFFECT OF STEEPING TEMPERATURE ON THE WET  
MILLING OF GRAIN SORGHUM

| Steep Temp. | Starch Recovered, d.b. | Protein in Starch, d.b. | Protein in Gluten, d.b. | Starch in Gluten, d.b. | Protein in Steepwater Solids | Viscosity of Starch, Scott |
|-------------|------------------------|-------------------------|-------------------------|------------------------|------------------------------|----------------------------|
| °F.         | %                      | %                       | %                       | %                      | %                            | sec.                       |
| 110         | 78.4                   | 0.78                    | 49.6                    | 33.8                   | 1.78                         | 115                        |
| 120         | 72.4                   | 0.77                    | 37.6                    | 51.0                   | 1.86                         | 105                        |
| 130         | 69.6                   | 0.86                    | 41.3                    | 46.4                   | 1.58                         | 86                         |
| 140         | 59.0                   | 0.61                    | 34.2                    | 55.8                   | 1.31                         | 71                         |
| 150         | 59.0                   | 0.79                    | 24.5                    | 67.2                   | 1.30                         | 55                         |

*Time of Steeping.* In the wet milling of corn on a laboratory scale, it had been determined that a steeping period of 24 hours was adequate when the operation was conducted batchwise. Several lots of grain sorghum were steeped for 24 and 71 hours and the grain then milled. It was found that the quality and recovery of starch, gluten, and germs were not improved by the longer period of steeping. As with corn, batchwise steeping for 24 hours in the laboratory method is satisfactory for the wet milling of grain sorghum.

*Pitch of Starch Table.* In the previous experiments sorghum mill starch was tabled under conditions which had been found to be satisfactory for the separation of cornstarch and gluten. The starch table was inclined at a pitch of  $\frac{1}{8}$  in. per 20 ft. of length which is the pitch used industrially by several processors of corn. When corn was wet milled in the laboratory equipment with the table set at this pitch, a good recovery of starch was obtained and the protein content of the starch was low, 0.30 to 0.40% (d.b.). In the experiments with grain sorghum it was noted that tabling was poor in some cases; the settled starch contained numerous pockets of gluten and the protein content

of the recovered starch was high, 0.7–1.0% (d.b.). In an attempt to improve the quality of the sorghum starch a few exploratory runs were made in which the sorghum mill starch was tabled with the unit set at a pitch of  $1\frac{3}{4}$  in. per 20 ft. The results were encouraging and a series of experiments was conducted to investigate the effect of this variable.

The grain was steeped for 24 hours at a temperature of 118°F. in water containing 0.25% sulfur dioxide. In order to expedite the tests the recovery of germs by flotation was eliminated. The steeped grain was ground twice and germs and fibers removed from the slurry by the regular screening operations.

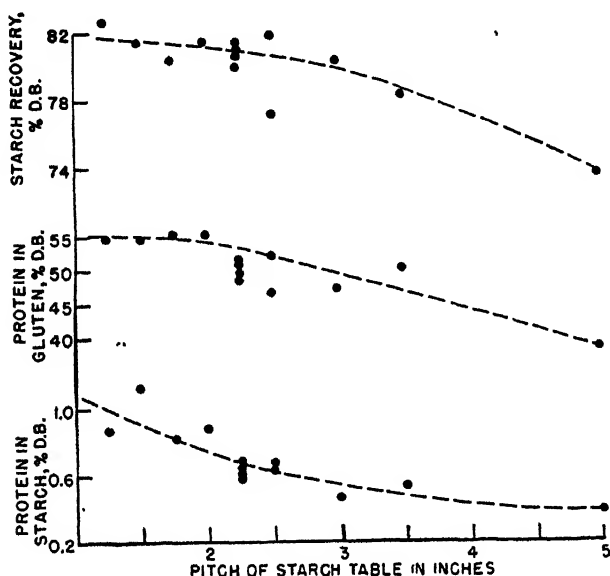


FIG. 6. The effect of table pitch on laboratory separation of starch, and protein in wet milled grain sorghum.

In the experiments the slope of the starch table was varied from  $1\frac{3}{4}$  in. to 5 in. per 20 ft. of table length. The results of the tests are shown graphically in Fig. 6. The correlation between pitch of table and starch recovery, protein in starch, and protein in gluten, is not precise, but a very definite trend is indicated. As the pitch of the table was increased, the protein contents of the starch and gluten, and the starch recovery decreased. The best starch contained 0.39% protein (d.b.) and was obtained when the table pitch was 5 in. per 20 ft. However, only 73.7% of the starch in the grain was recovered, and the comparatively large quantity of starch in the overflow from the table reduced the protein content of the gluten.

Based on the results of the tests it was concluded that for the wet milling of Martin sorghum, the starch table should be pitched at  $2\frac{1}{4}$ – $2\frac{1}{2}$  in. per 20 ft. of table length. Under these conditions the starch recovery was good and the protein content of the recovered starch, 0.6–0.7% (d.b.) was improved over results obtained in previous experiments.

It should be understood that optimum pitch of a starch table is not simply a function of the grain to be processed. The effect of the grain, or rather of the characteristics of the starch and gluten that it contains, is most important. However, other factors such as steeping conditions, milling of the grain, the rate at which mill starch is pumped to the table, and the physical characteristics of the table, have an influence on the recovery and quality of the starch.

*Wet Milling of Artificially Dried Grain Sorghum.* In 1947, the Midwest Research Institute, Kansas City, Missouri, was engaged by the Corn Products Refining Company to organize and supervise a series of drying experiments. A batch type of farm drier, designed by the Texas Agricultural Experiment Station, was used and operated by engineers from that station (10). The Northern Regional Research Laboratory entered into a cooperative agreement with the Corn Products Refining Company for the wet-milling tests.

Samples of Early Hegari and Martin were harvested at three moisture ranges, namely, low or 14 to 16%, medium or 17 to 20%, and high or 21 to 26%. Samples were dried with air at 125°, 150°, 175°, and 200°F. down to moisture ranges of 7 to 9% and 11 to 13%. One batch was dried with air at 230°F.; another was dried for  $\frac{1}{2}$  hour at 150°, then  $\frac{1}{2}$  hour at 230°F.; while a third sample had these second conditions reversed.

All the grain after drying graded No. 1, except for four samples which had a very slight toasted odor. The moisture content of the samples ranged between 10.1 and 12.3% at the time the samples were wet milled.

Four-pound samples of each batch of artificially dried grain were wet milled under identical operating conditions. Duplicates were run for each sample in the series. The grain was steeped for 24 hours at 118° to 120°F. with 2,800 cc. of a solution consisting of 0.20 gm. sulfur dioxide per 100 cc. of distilled water.

The steeped sorghum was milled according to the simplified procedure mentioned previously.

The specific gravity of the mill starch was adjusted to 6° Baumé (60°F), and the mixture then tabled at room temperature for the separation of starch and gluten. The table used was a painted 4-in.

channel iron, 20 ft. long, with a pitch of  $2\frac{1}{2}$  in. for its entire length. A nozzle was used to feed the mill liquor to the table at a rate of 295 cc. per minute.

The results of the investigation are given in Table IV.

In most of the tests with Martin variety sorghum, the grain milled well. The data shown in Table IV indicate that some damage was sustained by the samples of medium and low moisture content that were artificially dried to 7 to 9% moisture. This was revealed by the lower starch yields and increases in the protein content of the starch. All of the samples harvested with a high moisture content, processed and tabled very well. Evidently these samples were not damaged during the drying operation. No relationship was noted between the temperature of drying and apparent damage. It was observed that the samples of artificially-dried Martin variety sorghum were harder after steeping than naturally dried sorghum of the same variety and, therefore, required more power for grinding.

All of the samples of Early Hegari milled well, indicating they were not injured by the drying. However, samples of this variety harvested with a low moisture content were dried only to 11 to 13% moisture and not to 7 to 9%. It is possible that Early Hegari may suffer damage, as did Martin, if harvested when its moisture content is low and dried to 7 to 9% moisture.

*Pearled Grain Sorghum.* One difficulty in wet milling some varieties of grain sorghum is that the pigment from the outer layers of the kernel permeates the grain during steeping, resulting in the production of colored starch. The product may be tinted shades of yellow, pink, or purple, depending upon which variety is processed. Although in some varieties the highly colored undercoat is absent at maturity, enough coloring matter may be present in the outer layers of the bran to tint the final starch. The glumes also are a possible source of color, but most of these are removed when the grain is cleaned. Sorghum breeders are striving to perfect varieties for industrial use that are free from coloring matter. Fortunately, the combine varieties, Martin and Early Hegari, give starches that are not too highly colored.

It was thought that, if the coloring layers of the sorghum were removed prior to steeping, a whiter starch could be made. With this in mind, tests were conducted to compare the wet milling of whole and pearled sorghum. Pearling was accomplished by passing the grain through a laboratory Strong-Scott barley pearler in which 100 gm. portions of grain were retained for 10 to 15 seconds. This resulted in the removal of 15 to 20% of the weight of the grain as bran and material which passed through a 12-mesh screen.





Table V gives the composition of the whole and pearled sorghum, along with the fraction removed by pearling which is referred to as bran.

The samples of whole and pearled grain were processed in the laboratory by the wet-milling procedure illustrated in Fig. 2.

The starch recovered from the pearled grain was whiter than starch made from the whole sorghum. However, it is questionable that this procedure is economically feasible since a substantial quantity of starch is lost in pearling.

Considerable work has been done by F. A. Kummerow *et al.* (8) at Kansas State Agricultural College on the recovery and properties of a carnauba-like wax which is contained in the outside layers of grain sorghums. If this wax can be recovered economically from the bran fraction, it may be profitable to pearl the grain before it is wet milled.

TABLE V  
CONSTITUENTS OF WHOLE AND PEARLED MARTIN GRAIN SORGHUM

|                    | Whole | Pearled | Bran |
|--------------------|-------|---------|------|
| Moisture %         | 10.0  | 10.7    | 8.8  |
| Starch % d.b.      | 68.9  | 74.3    | 35.8 |
| Protein % d.b.     | 15.0  | 15.3    | —    |
| Oil and Wax % d.b. | 3.7   | 2.8     | 7.0  |

Only a few exploratory tests were conducted on the recovery of the wax fraction. Hexane extraction of the bran fraction yielded 5 to 10% of its weight as a crude wax of a brownish green color. Hexane and benzene were the most suitable of the various solvents tried for this purpose. Heptane, iso-heptane, and carbon tetrachloride were difficult to recover. Pigments were removed along with the crude wax when ethyl acetate, or ethyl or iso-propyl alcohols were used. The crude wax upon extraction with acetone yielded a light tan, brittle wax with a melting point of 81°C. A soft fraction consisting mainly of higher alcohols was obtained from the solvent. The brittle wax possibly could be used as a substitute for carnauba wax.

### Discussion

It was found that grain sorghum could be processed by a procedure similar to that used industrially for the wet milling of corn. Sorghum starch is not affected by concentrations of sulfur dioxide in steepwater as high as 0.25% at temperatures under 120°F. These are practical operating conditions. It has been shown that grain sorghum can be steeped batchwise in 24 hours. This is a laboratory procedure and the result cannot be transferred directly to plant operation. However,

since corn is steeped satisfactorily in the laboratory in 24 hours, it is assumed that the conditions employed in the countercurrent, multiple contact system for steeping corn on a plant scale will be satisfactory for grain sorghum.

No results have been reported on the recovery of germs from grain sorghum since the operation is almost impossible to conduct quantitatively in the laboratory. However, it was observed that germ recovery was easier and more complete when the concentration of sulfur dioxide in the steepwater exceeded 0.20%.

It appears to be more difficult to separate sorghum starch and gluten than the same materials obtained from corn. It was found that the pitch of the starch table was a critical factor in the preparation of sorghum starch of low protein content. Starch produced from artificially dried grain sorghum contained as little as 0.3% (d.b.) of protein. However, in general, protein contents ranged from 0.4 to 0.7% (d.b.).

If carnauba-like wax can be recovered from grain sorghum economically it may be feasible to pearl the grain before it is wet milled. A whiter starch is obtained and the processing is simplified to some extent since most of the fiber is removed during pearling.

#### Acknowledgment

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## EXTRACTION AND ELECTROPHORETIC ANALYSIS OF THE PROTEINS OF CORN <sup>1</sup>

JOSEPH F. FOSTER, JEN TSI YANG, AND N. HENRY YUI <sup>2</sup>

### ABSTRACT

Practically quantitative extraction of the proteins of corn can be effected with dilute aqueous solutions of alkyl benzene sodium sulfonate containing 0.2% bisulfite. In the absence of the reducing agent extraction is limited to about 80%. The whole corn protein extracts are reasonably clear, even following removal of the excess detergent by dialysis against water. The extraction with other media is re-examined, it being found that water removes 11% of the protein, 5% sodium chloride solution 22%, pH 10 carbonate buffer 30%, and pH 7.8 phosphate approximately 20%, all at 2°C. Aqueous ethanol (73%) at room temperature removes approximately 40%. In general extraction with the various media appears to be additive although the yield in the sequence water-salt-carbonate-detergent is not quantitative. Phosphate ion appears to have a very significant insolubilizing action on the proteins, alcohol a somewhat less effect.

Electrophoretic analysis of the detergent extract indicates the presence of at least eight components. Three of these are identified with zein, the others being concentrated in the aqueous extracts.

The question of the overall protein composition of corn is not well clarified. In large part this can be attributed to the fact that the major protein constituent, zein, is soluble only in media which render application of the usual analytical procedures, particularly electrophoretic analysis, difficult or impossible. The amount of zein present and its relationship to the other proteins in corn is in doubt. Thus, it is not clear whether there is a sharp line of demarcation between the proteins or whether there exists a spectrum of proteins ranging from the typical prolamine type to the alkali-soluble glutelins.

It has not heretofore been possible to extract all of the protein of corn in a single extractant or even with a combination of several extractants. Perhaps the nearest approach lies in the work of Nagy, Weidlein and Hixon (4). These workers were able to extract as much as 85% to, in some cases, 90% of the protein of corn using a combination of salt solution, alcohol, and alkali. In this study the workers also observed an unusual solubilizing effect on the corn proteins due to the synthetic detergents. However, they were not able to extract over 60

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to 70% of the protein with the detergent solutions under the conditions they used.

It is now known that zein is soluble in dilute aqueous solutions of soaps and synthetic detergents and the physical chemistry of such solutions has recently been studied by one of us (2), it being shown that in such solutions the zein is present as a very stable zein detergent complex. This raises the possibility of extracting all of the corn protein in a single aqueous medium which could be subjected to electrophoretic analysis.

The present paper demonstrates that substantially quantitative extraction can be attained using dilute aqueous detergent solution buffered at pH 10 containing in addition a small amount of reducing agent such as sodium bisulfite. At least eight electrophoretic components are present. Extraction with such media as water, salt solution, phosphate buffer, carbonate buffer and aqueous alcohol has also been re-examined and electrophoretic analyses made.

### Materials and Methods

These studies were carried out on a commercial cornmeal ground to approximately 60 mesh. This sample contained 1.66% Nitrogen on a dry basis corresponding to a protein content of 10.4%. In most of the extraction studies the meal was first defatted by stirring with 99% isopropyl alcohol at room or cold room temperature. Usually 20 gm. samples were stirred twice for approximately 24 hours periods with 100 ml. of the alcohol, and separated by centrifugation. Fat, determined by evaporation of the solvent and weighing of the dry residue, amounted to 4.5–4.7% when extracted at cold room temperature (2°C.) and 5.5 to 5.7% at room temperature. Nitrogen was also determined on the alcohol extracts and amounted to 0.8 to 1.1% of the total nitrogen.

The detergent used was the commercial Santomerse No. 3,<sup>3</sup> an alkyl benzene sulfonate. In some experiments this material was purified by precipitation from ethanol, but with no improvement in extraction.

*Extraction Procedure.* In early studies extraction was carried out simply by slow mechanical stirring in a beaker. In later work, for reasons mentioned below, shaking was substituted for stirring. In this case the extractions were carried out in 250 ml. centrifuge tubes, the volume of extracting fluid being usually 100 ml., and separation made by centrifugation in an International centrifuge at approximately 2,000 r.p.m. Most extractions were carried out in parallel at room temperature (25–30°C.) and in the cold room (2°C.).

*Nitrogen Analysis.* The amount of protein extracted was deter-

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<sup>3</sup>Supplied by the Monsanto Chemical Co.

mined by nitrogen analysis on the extracts using the Pregl micro technique, with selenium oxychloride as catalyst. Analysis of the corn and of the residue following extraction was by the conventional macro procedure using cupric selenite as a catalyst.

*Dialysis.* Dialyses were carried out in cellophane tubing<sup>4</sup> using continuous mechanical agitation. For electrophoresis the dialysis time was about 24–48 hours against the appropriate buffer. For removal of excess detergent, dialysis was against five or six changes of water, 12 to 24 hours each. Following dialysis the extracts were concentrated by lyophilization, where necessary, to a protein concentration of at least 0.6% for electrophoretic analysis. In case of the non-detergent extracts solutions were usually somewhat turbid following concentration and centrifugation was frequently necessary to clarify them sufficiently for electrophoretic analysis.

*Electrophoretic Analysis.* The electrophoretic studies were carried out in a standard double-length cell of the Tiselius type. The apparatus used and method of operation were the same as previously described by one of us (1).

### Results and Discussion

*Extraction with Aqueous Detergent. General Considerations.* The first extractions carried out were with unbuffered detergent solution. In such cases the pH of the detergent, about 8.3, dropped to about 7.0 upon contact with the corn. Extraction yields under such conditions were very poor. Furthermore, it had been found in the case of corn gluten that maintenance of the pH above 9.0 greatly facilitated extraction (7). Therefore, the detergent solutions were buffered with sodium carbonate-bicarbonate at a pH of 10 to 11 in all detergent extractions reported in this paper. The actual pH following extraction ranged from about 9.0 to 10.0.

The first phase of the investigation was concerned with the optimal mechanical conditions for rapid extraction. In runs Nos. 6 and 7 (Fig. 1), 38.0 gm. samples of corn were extracted with a single batch of 200 ml. of 1% detergent solution using vigorous mechanical stirring. Aliquots were removed each day, centrifuged, and the supernates analyzed for nitrogen. Extraction is evidently very slow after the first day. In runs Nos. 8 and 9 (Fig. 1), 18 gm. samples were extracted with 100 ml. portions of 1% detergent for 24 hours, with removal of extract and washing of the residual corn with carbonate buffer (pH 10.3) following each such period. This procedure is seen to be somewhat more efficient.

In runs Nos. 13 and 14 (Fig. 1), extraction was first carried out with carbonate buffer (pH 10.3), 38.0 gm. samples being twice extracted

<sup>4</sup> Supplied by The Visking Corp., Chicago. \*

with 200 ml. of buffer each time. Each extraction was followed for two to three days. Following this, the residue was extracted twice with 200 ml. portions of detergent (1%), again for two to three days. In all cases extraction can be considered to be substantially complete after 24 hours. Furthermore, the second portion of a given extractant accomplishes relatively little, except in the case of the second extraction with alkaline buffer at room temperature.

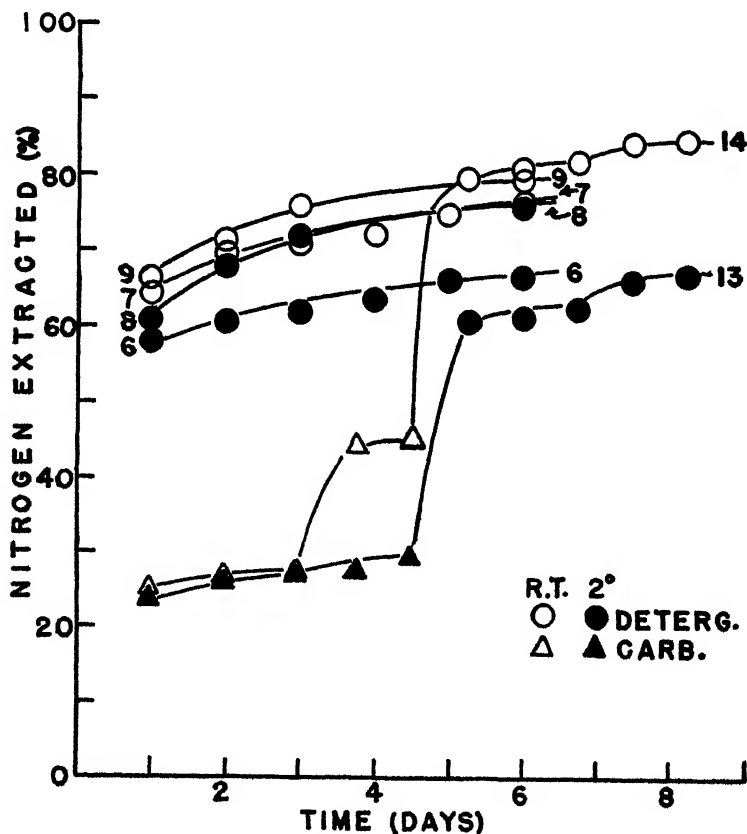


FIG. 1. Extractions with detergent and with carbonate buffer without bisulfite.

This anomalous result with carbonate was observed repeatedly. It was concluded that the effect must be due to microbiological action since it occurred only at room temperature and did not occur in the detergent extractions. In a few extreme cases obvious evidences of deterioration, such as the odor of hydrogen sulfide, were detected. Addition of an effective antiseptic minimized this effect although there were always some doubts as to the validity of results carried out in

alkaline, detergent-free media at room temperature over a period of several days. The antiseptic used in all room temperature work reported below, with the exception of detergent extractions where no evidences of decomposition were ever observed, was *p*-chlorothymol (usually in the amount of 5 ml. of a saturated aqueous solution per 100 ml. of extracting solution).

In the early extractions the extracts were frequently very cloudy following centrifugation. It was concluded that this was in part due to fat, in part due to suspended starch particles. Substitution of either an end to end agitation of the tubes in a horizontal position or end over end rotation yielded equally rapid extraction and much clearer extracts. Use of defatted corn further improved the situation. All subsequent results were obtained with these modifications.

*Extraction with Aqueous Detergent Solution.* It is apparent from Fig. 1 that extraction with buffered detergent solution or even with alkaline buffer followed by detergent, does not effect quantitative removal of the proteins. The curves appear to level off below 80% extraction and attainment of even this yield is a slow, tedious process. Furthermore, the extracts in these studies were very cloudy, as previously mentioned, and clarification resulted in a loss of as much as 20% of the extracted nitrogen so that even the 70 or 80% figure is high.

Attempts were made to extract the residual nitrogen using aqueous alcohol with and without added sodium acetate at room temperature and at elevated temperature. At room temperature only some 2.0% additional nitrogen could be extracted, at 60° somewhat more; however, the total yields were still not over 80%.

*Detergent Plus Reducing Agent.* The low yields of protein obtained with the alkaline detergent solutions were distinctly disappointing, particularly in view of the fact that prior to this it had been found possible to extract the protein of commercial corn gluten quantitatively with such a medium (6). It seemed possible that this difference in extractability might be due to the reducing action of the bisulfite steep used in the commercial wet-milling process. Also it is well-known that reducing media facilitate the solubilization of other tissue proteins, notably keratins (3). Therefore, experiments were conducted in which sodium bisulfite in the amount of 0.2% was added to the detergent extractant solutions.

Some results of direct detergent extraction in the presence of bisulfite are shown in Fig. 2. In runs Nos. 29 and 30, 20 gm. samples of defatted corn were extracted repeatedly for 2 day periods with 100 ml. portions of 2.0% detergent containing also 0.2% bisulfite. In runs Nos. 33 and 34, conditions were the same except that the detergent solution used was only about 0.4% (saturated at the cold room tempera-



ture). Runs Nos. 29 and 33 were in the cold room, Nos. 30 and 34 at room temperature. Extraction is obviously much improved and seems to approach 100%.

In Fig. 3 are shown results of experiments in which detergent extraction followed either water or alkaline buffer extraction. In all of these runs conditions were the same during the detergent extractions, i.e., 20 gm. corn extracted repeatedly for 48 hour periods with 100 ml.

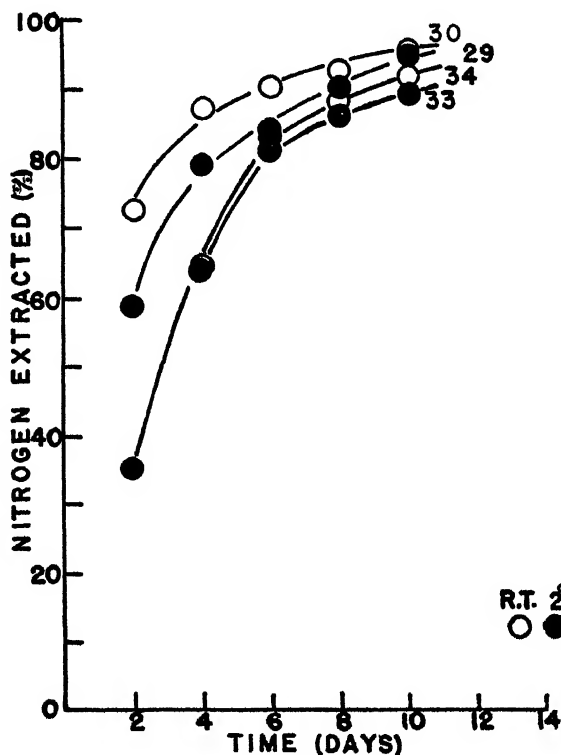


FIG. 2. Extractions with detergent plus bisulfite.

portions of 2% detergent plus 0.2% bisulfite. Removal of the water or alkali soluble protein appears to have no marked influence on the course of the detergent extraction.

The fact that much less nitrogen is extracted in the early stages of run No. 33 as compared to No. 29 (Fig. 2) shows that the excess undissolved detergent in the cold room extractions is effective. This might be expected since presumably the excess dissolves to maintain a saturated system as detergent is combined with protein. At the end of five extractions (10 days) the amount of nitrogen extracted in runs Nos. 33

and 34 is comparable to that in Nos. 29 and 30 in spite of the fact that only 20% as much detergent is used in Nos. 33 and 34. This is of considerable interest and indicates that the ratio of detergent to protein is the limiting factor. Thus the amount extracted in extractions Nos. 1 and 2 is limited by the low detergent concentration in runs Nos. 33 and 34 but is made up in later extractions.

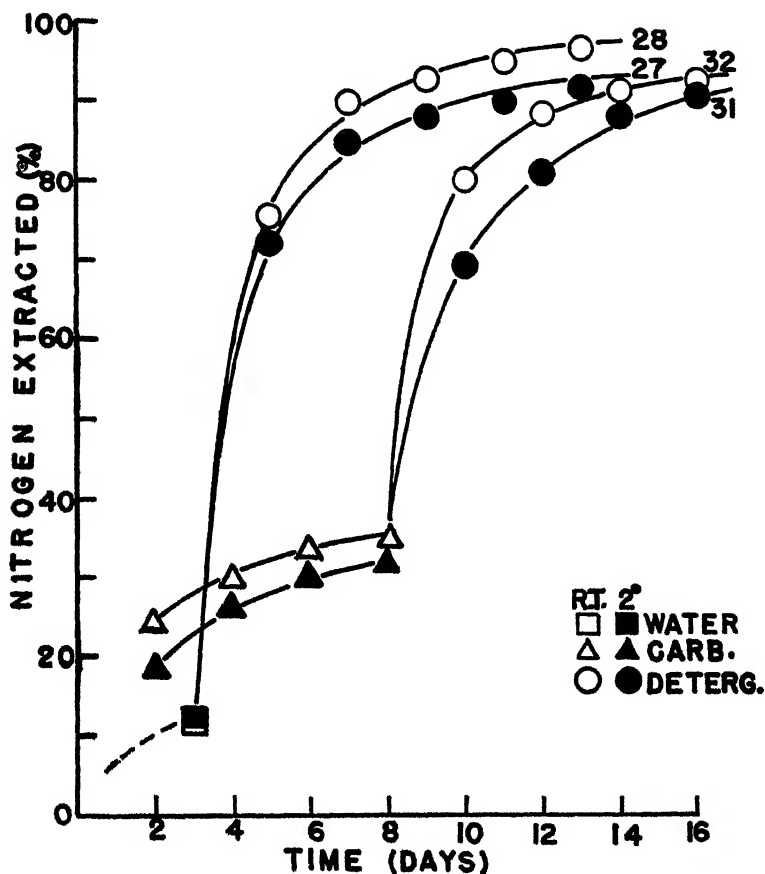


FIG. 3. Extractions with water or carbonate buffer followed by detergent. Bisulfite present in detergent and carbonate extractions.

This result suggested the possibility of reducing the detergent concentration in successive extractions as a means of economizing on detergent without sacrificing on yield or extraction rate. This proved perfectly feasible. In one such experiment a 20 gm. sample of corn was extracted five times as in run No. 29 except that the extracting medium consisted of 1.0, 0.5, 0.3, 0.2, and 0.1% detergent. The

protein extracted was actually slightly higher than in run No. 29. Extraction was 90.0% complete after four extractions. In other words 1.8 gm. of protein was extracted with only 2.0 gm. of detergent. In this experiment the bisulfite concentration was also reduced in successive extractions, from 0.10% down to 0.01%. The ratio of detergent to protein is not the sole factor, however. In a parallel experiment to this a sample of only 5 gm. was extracted with 0.3, 0.2, 0.1, and 0.05% detergent, a total of 0.65 gm. of detergent to only about 0.5 gm. protein. Extraction was very poor, about 76%.

A final water wash following three or four detergent extractions is as effective as another detergent extraction. Presumably this results from solution of protein-detergent complex already formed.

Thus it is seen possible to extract in the form of clear solutions 90% or more of the protein of corn, at 2°C. using little more than 1 gm. of detergent per gm. of protein. Actually the extraction is somewhat better than this since the micro-Kjeldahl values tend to be a little low and some mechanical loss is unavoidable. Electrophoretic analyses on such extracts are shown in Fig. 5 and are discussed below.

Approximately 12% of the nitrogen in these extracts was found to be dialyzable in several experiments and is to be presumed nonprotein in character.

*Other Extractants. Alkaline Buffer.* Direct extraction with pH 10.3 carbonate buffer in the cold room removed 27 to 29% of the protein (Fig. 1, Exp. 13). The same total is obtained when carbonate follows water and salt extraction. The problem of decomposition in this buffer at room temperature has been previously mentioned. However where adequate protection against microbiological growth was used, the curves at room temperature also appeared to flatten out at about 30%. Alkaline buffer plus bisulfite is appreciably more efficient, 30 to 35% being extracted (Fig. 3, runs Nos. 31 and 32). Exposure to the alkaline buffer has no adverse effect on the later extraction with other solvents.

*Water.* Water extracts approximately 11% of the protein, either at room or cold room temperature (Fig. 3, runs Nos. 27 and 28 plus many other data not reported here). Extraction with water does not have any adverse effect upon later extractions with other media.

*Salt Solution.* Extraction with 5% aqueous sodium chloride yields 22% of the protein, following water extraction approximately 11% (a total of 22% with the two extractants). Carbonate buffer following water and salt extraction removes an additional 4.9%, the total agreeing with that obtained by direct alkaline buffer extraction. As seen in Fig. 4, run No. 44, detergent extraction following water, salt, and carbonate is not very effective. As previously pointed out neither water nor carbonate alone interfere with subsequent detergent extraction and

it has also been found that salt followed by detergent gives substantially quantitative extraction.

*Phosphate Buffer.* Direct extraction with phosphate buffer removes only about 20% of the protein (Fig. 4, runs Nos. 21 and 22). Furthermore the residual protein is rendered much less soluble as indicated by the fact that alkaline buffer following phosphate removes only about 3

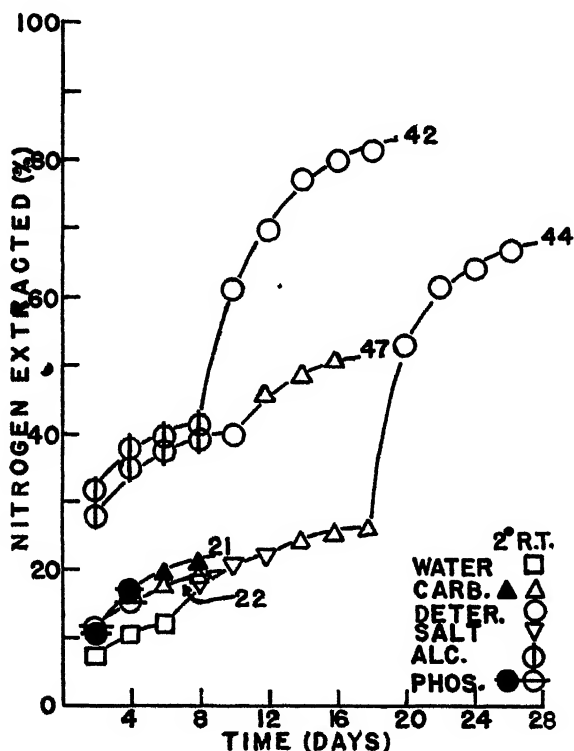


FIG. 4. Extractions with water, carbonate buffer, 5% sodium chloride, 73% ethanol, phosphate buffer, and detergent. Bisulfite present in detergent extraction.

to 5.0% additional protein and extraction with other media is greatly reduced. Detergent without reducing agent following phosphate plus alkaline buffer yielded only 28%. Even with reducing agent extraction is far from complete once the corn has been exposed to phosphate. Exhaustive washing of the residue with water to remove phosphate ions improved this situation only slightly. It appears that phosphate may have an insolubilizing action on the corn proteins.

*Aqueous Alcohol.* Direct extraction with aqueous alcohol yields about 40% of the protein at room temperature in the presence of sodium acetate. This is in agreement with the findings of Hixon *et al.* (4). More can be extracted at elevated temperature. Extraction with

alcohol somewhat inhibits subsequent extraction with detergent (Fig. 4, run No. 42). Also extraction with alkaline buffer following alcohol appears to be inhibited (Fig. 4, run No. 47). This effect is probably merely one of rate since these curves have not leveled off. In at least one experiment it was found possible to extract a total of over 90%

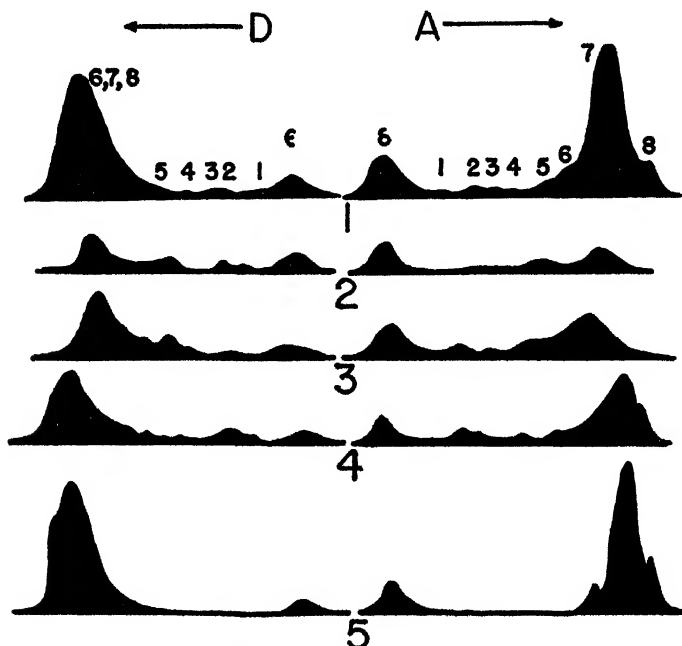


FIG. 5. Electrophoretic analyses in the presence of detergent. Run No. 1, whole-protein, 1.0% protein, 135 minutes at 6.3 volts/cm. Run No. 2, water extract, 0.4% protein, 106 minutes at 6.0 volts/cm. Run No. 3, sodium chloride extract, 0.5% protein, 102 minutes at 6.1 volts/cm. Run No. 4, carbonate buffer extract, 0.6% protein, 135 minutes at 6.4 volts/cm. Run No. 5, 73% ethanol extract, 0.8% protein, 143 minutes at 6.1 volts/cm. The buffer in all cases was carbonate-bicarbonate, pH 10.3, ionic strength 0.26.

of the protein with alcohol followed by detergent. However, it was also noted that such extracts were turbid whereas the direct detergent extracts were quite clear. Thus it seems certain that the alcohol has an insolubilizing action on some of the proteins present in corn.

*Electrophoretic Analysis of Extracts.* Electrophoretic analyses of water, salt and carbonate buffer extracts have been carried out. The extracts are always very inhomogeneous, containing components ranging in mobility from about 2-3 to  $10 \times 10^{-6}$  cm.<sup>2</sup> Volt<sup>-1</sup> Sec.<sup>-1</sup>. It is concluded that such extractions are not particularly selective. Furthermore the analyses are complicated in that the extracts, being very dilute, must be concentrated prior to electrophoresis and there is invariably loss of some protein during this step.

The detergent extracts remain clear following concentration and can be subjected to electrophoresis conveniently. However, in view of the known tendency of proteins to form complexes with anionic detergents the results can be interpreted only with extreme caution (2,5). Results of a typical run on the whole-protein extract from which excess detergent has been removed by prolonged dialysis are shown in Fig. 5. Usually about eight components are distinguishable and these have been numbered in the figure in order of increasing mobility. The mobilities are somewhat variable, probably due to variations in the extent of removal of detergent, but usually range from about  $3.0$  to about  $11\text{--}12 \times 10^{-5} \text{ cm.}^2 \text{ Volt}^{-1} \text{ Sec.}^{-1}$  under conditions similar to those used in Fig. 5. (The detergent itself yields a single peak with mobility of  $16\text{--}18 \times 10^{-5}$  under these conditions.) It seems probable that this represents a minimum number of components since combination with detergent has the effect of increasing total net charge, thereby causing the proteins to lose their electrophoretic identity. This has been verified by suitable control experiments on mixtures such as zein plus ovalbumin run in the presence of detergent.

A typical electrophoretic run on the 73% ethanol extract under conditions similar to those used in the case of the whole protein is also reproduced in Fig. 5. It is interesting that only the three components of highest mobility (6, 7, and 8) appear in such extracts in appreciable quantity. Conversely, the water, salt, and carbonate buffer extracts are considerably enriched in the slower moving components when analyzed in the presence of minimal amounts of detergent (Fig. 5).

The fact that slow moving components are obtained at all, in the presence of detergent, would seem to indicate that the more water soluble components give up their detergent upon dialysis more readily than zein. If this is true, the method might have value as an empirical analytical method for comparing, for example, different strains of corn. Obviously much further work will be necessary before this is possible. In particular precision methods for determining the residual bound detergent are needed. Further work along these lines is in progress.

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# IMPROVED TECHNIQUES FOR STUDY OF THE HYDROLYSIS OF AMYLOSE BY MALT ALPHA-AMYLASE<sup>1,2</sup>

R. J. DIMLER, R. C. BACHMANN AND H. A. DAVIS

## ABSTRACT

Amylose solutions of 3 to 4% concentration can be hydrolyzed with malt alpha-amylase without retrogradation if a temperature of 70°C. is used. This high temperature, together with the presence of 0.1% calcium chloride in the amylose solution, minimized possible action of traces of beta-amylase which might have been in the alpha-amylase preparation. The iodine color of the hydrolysis solution reached the dextrin end-point at about 30% conversion and the achromic point at about 45% conversion to apparent maltose. After the hydrolysis was stopped by addition of acetic acid and the solution deionized, the products could be partially fractionated by alcohol precipitation. Paper chromatography showed that the fractions were still very heterogeneous. At the achromic point, the hydrolysis products were found to consist of at least 10 reducing compounds, including glucose and maltose and, presumably, the homologous series of oligosaccharides up to a degree of polymerization of 10 glucose units.

The first study of the hydrolysis of a relatively pure amylose by malt alpha-amylase was described by Meyer and Bernfeld (7). Subsequent work by Myrbäck and coworkers (9, 13) included fractionation by alcohol precipitation of the hydrolysis products at different stages of hydrolysis of the amylose. Data on rates of hydrolysis and on distribution of molecular sizes were used as a basis for theories on the mechanism of alpha-amylase action. Bernfeld and Studer-Pécha (4) have described experiments showing the effect of retrogradation of amylose on the rate and extent of hydrolysis by alpha-amylase. These authors considered the conclusions of Myrbäck unreliable because they were based on experiments in which retrogradation must have occurred (see also Meyer, (8)). The possibility that the fractionation methods used by Myrbäck and coworkers give fractions which are still quite heterogeneous is indicated by the carbon-column adsorption studies of Tiselius and coworkers (16, 17) on fractions furnished by Myrbäck, apparently mainly from starch hydrolyzates.

Our interest in the mode of action of malt alpha-amylase on amylose has led to the development of the methods of operation described

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in this paper. Particular attention was given in this work to methods of minimizing the danger of retrogradation, increasing the usable amylose concentration in order to facilitate the preparation of larger quantities of materials, and evaluating the effectiveness of the fractionation methods.

### Materials and Methods

*Malt Alpha-Amylase.* The preparation of the malt alpha-amylase was based on the work of Kneen, Sandstedt and Hollenbeck (6) on the stability and precipitability of the enzyme. Ground barley malt was extracted with five parts of 0.2% calcium chloride solution at pH 6.0, the filtered extract heated at 70°C. to inactivate beta-amylase, and the alpha-amylase precipitated from the cooled solution by half-saturation with ammonium sulfate. This precipitate was converted to a stable, easily soluble form by dissolving it in 0.2% calcium chloride solution and drying it cryoscopically. The resulting product, representing about 60% of the alpha-amylase originally extracted from the malt, usually had a potency of about 3,000 units per gram by the Sandstedt, Kneen and Blish (11) method.

*Amylose.* The amylose was prepared from potato starch by the butanol fractionation method of Schoch (12). The recrystallized amylose had an iodine sorption of 201 mg. of iodine per gram by the potentiometric titration method of Bates, French and Rundle (2) as modified by Wilson, Schoch and Hudson (18). In comparison, the maximum iodine affinity of repeatedly recrystallized potato amylose was about 210 mg. of iodine per gram. The amylose either was used as the wet amylose-butanol complex or was dried in such a way as to avoid retrograded or horny products.

*Analytical Methods.* Reducing power was determined by the method of Somogyi (14). The results were calculated as maltose hydrate.

The qualitative test for butanol in aqueous solution depended upon formation of butyl acetate which was detected by its odor. To a mixture of 2 ml. of the aqueous solution and one drop of glacial acetic acid was added 1 ml. of concentrated sulfuric acid. The heat of mixing aids in the esterification of the butanol and in the volatilization of the butyl acetate. As little as about 0.25% butanol could be detected in this way.

The color of the hydrolyzate with iodine was determined by using the iodine solution and the proportions prescribed for the Sandstedt, Kneen and Blish alpha-amylase determination (11). Measurements were made with a Coleman spectrophotometer, the comparison being either with the iodine blank at 500 m $\mu$  or with the inorganic standard of Olson, Evans and Dickson (10) at 635 m $\mu$ .



Qualitative analysis of fractions by paper chromatography was done by the multiple development method of Jeanes, Wise and Dimler (5), using a 1:1:1 mixture of fusel oil, pyridine, and water and four ascending movements of the solvent. The position of the sugars was determined by spraying the dried paper with an alkaline solution of 3,5-dinitrosalicylic acid. The initial spot contained about 0.4 mg. of carbohydrate, placed on the paper by application of the necessary number of 1- $\lambda$  drops to the same spot with drying between each application.

*Preparation of Amylose Solution.* A 3% solution of potato amylose was prepared by adding a slurry of 60 gm. amylose (dry basis) in 400 ml. *n*-butanol with stirring to 1.6 l. of 0.1% calcium chloride solution at 90°C. To eliminate the possibility of "skin formation" during removal of the butanol it is advantageous to have the solution in a 3-necked, 3-l. round-bottom flask fitted with a distilling head, a stirrer, and a graduated dropping funnel. When solution was complete the butanol was removed by distillation until a negative test for butanol was obtained in the distillate. This usually required distillation of about 1 l. of the mixture. Nearly complete removal of butanol is essential to avoid inactivation of the alpha-amylase. The volume of solution in the flask was kept constant by the continuous addition of water during the distillation.

*Hydrolysis of Amylose.* The flask containing the aqueous solution of amylose (pH about 6.0) was transferred to a 70°C. constant temperature bath. When the temperature of the stirred solution had dropped to between 70° and 75° the alpha-amylase, dissolved in 0.2% calcium chloride, adjusted to pH 6.3 with dilute ammonium hydroxide and preheated 3 minutes in the 70°C. bath, was added. As an example, a total of 10 units (Sandstedt, Kneen and Blish) of amylase per gram of amylose was used, about half of the enzyme being added initially, one-third after 15 minutes, and the remainder in two equal portions at 15-minute intervals. In this case the achromic point was reached in about 70 minutes. Hydrolysis was stopped by adding 100 ml. of 0.1 *N* acetic acid and holding the acidified (pH 4) solution at 70°C. for 20 minutes before cooling it in an ice bath. The incremental addition of enzyme was used in most cases to more or less compensate for some inactivation of the alpha-amylase at 70°C. Addition of all the enzyme at the beginning of the hydrolysis gave similar results except for requiring about 135 minutes to reach the achromic point.

*Preliminary Alcohol Fractionation of Products.* The cold hydrolyzate, after being supercentrifuged to remove a slight precipitate, was deionized by passage through columns of cation and anion exchange resins. The solution was concentrated to 180 ml. and added very

rapidly to 3.4 l. of absolute ethanol with stirring to precipitate a fraction insoluble in 95% (v/v) ethanol. After the mixture had been stirred for 18 hours to insure equilibrium, the precipitate was separated by decantation and centrifugation. This fraction was dissolved in 180 ml. of water and reprecipitated from 95% ethanol. It was then fractionated in 85% (v/v) ethanol by dissolving the centrifuged or filtered precipitate in 180 ml. of water and adding it to 1 l. of absolute ethanol. After being stirred overnight the slightly cloudy supernatant was decanted from the sirup which had separated. The fraction insoluble in 85% ethanol was obtained as a dry powder by dissolving the sirup in 90 ml. of water, adding the solution to 3 l. of absolute ethanol with stirring, separating the precipitate by filtration, washing it twice by resuspension in absolute ethanol, and drying it over calcium chloride in a vacuum desiccator. The fraction soluble in 85% and insoluble in 95% ethanol was isolated by concentrating the solution in 85% ethanol to dryness and converting the residue to a dry powder in the same manner used for the fraction insoluble in 85% ethanol. The fraction soluble in 95% ethanol was obtained as a sirup by concentrating the combined 95% ethanol solutions.

### Results and Discussion

A difficulty often encountered in working with solutions of amylose is the ease with which retrogradation occurs. In the present studies the relatively good stability of alpha-amylase at high temperatures permitted taking advantage of the fact that the rate at which amylose retrogrades decreases with increase in temperature. In earlier hydrolyses at 30° to 40°C. even 1% solutions frequently retrograded. At 70°C. the use of 3% solutions of amylose proved quite practicable, while some trials were successful with 4%, and slightly higher, concentrations. No cloudiness attributable to retrogradation was observed, although the possibility of formation of invisible submicrons (4, 8) was not excluded.

Retrogradation presumably could be minimized also by use of the procedure described by Bernfeld and Gürtler (3) for hydrolysis with beta-amylase. They added an alkaline solution of amylose to a buffered solution of an excess of enzyme. Such a procedure would not be suitable for the study of intermediate stages of hydrolysis, however, because of the differences in the period of hydrolysis of the various portions of the amylose solution.

With the use of 70°C. for the hydrolysis, the inclusion of calcium chloride for stabilization of the alpha-amylase (6) became desirable. This combination of high temperature and presence of calcium chloride offers the further advantage of providing unfavorable conditions for

beta-amylase (6), thereby minimizing the possibilities of hydrolytic action by traces of beta-amylase which might have remained in the alpha-amylase preparations.

The hydrolysis at 70°C. followed essentially the same course of increase in reducing power as reported by Myrbäck for malt alpha-amylase and by Alfin and Caldwell (1) and by Swanson (15) for pancreatic and salivary alpha-amylase, respectively. The initial rapid increase in reducing power was followed, after about 40–45% conversion to apparent maltose, by a much slower hydrolysis.

The progress of the hydrolysis during the last part of the rapid phase was conveniently followed by determining the color given by the addition of samples of the hydrolyzate to dilute iodine solution. The achromic point occurred regularly at about 45% conversion to apparent maltose. The spectrophotometrically measured transmission of the mixture at 500  $m\mu$  became constant at about 95% of the transmission of the iodine blank, apparently as a result of interaction between the iodine and substances in the enzyme preparation.

Another familiar point in the series of iodine colors is the end-point of the alpha-amylase determination (11) at which a red coloration is obtained. The extent of hydrolysis was approximately 30% when the transmission of the iodine-hydrolyzate mixture was the same as that of the inorganic standard (10) at 635  $m\mu$ . The test with iodine, therefore, provides a very useful guide for determining when to stop the reaction for these two approximate extents of hydrolysis in the phase of rapid hydrolysis.

Acidification of the hydrolysis mixture was adopted as a more instantaneous method of stopping the reaction than heating the mixture to boiling. This is particularly true when several liters of solution are involved and when the hydrolysis is to be halted at intermediate points in the rapid phase.

The acetic acid was removed along with the calcium chloride by the ion exchange resins. The removal of calcium chloride before alcohol precipitation of the products was found to be essential. Notwithstanding the solubility of calcium chloride in alcohol, the precipitated carbohydrates contained considerable ash if the solution had not been deionized.

TABLE I

| <i>Fraction</i>              | <i>Yield<br/>%</i> | <i>Average D. P.<sup>1</sup><br/>glucose units</i> |
|------------------------------|--------------------|----------------------------------------------------|
| Sol. 95% EtOH                | 40                 | 2.8                                                |
| Insol. 95%, Sol. 85% EtOH    | 16                 | 6.3                                                |
| Insol. both 95% and 85% EtOH | 44                 | 7.0                                                |

<sup>1</sup> D. P. indicates Degree of Polymerization or number of glucose units per molecule.

Fractionation of the hydrolyzate by precipitation with 95% and 85% (v/v) ethanol was satisfactory only for a preliminary separation of the products into somewhat less complex mixtures. The results shown in Table I are for such a fractionation of the products from a hydrolysis which had been carried to just past the achromic point and

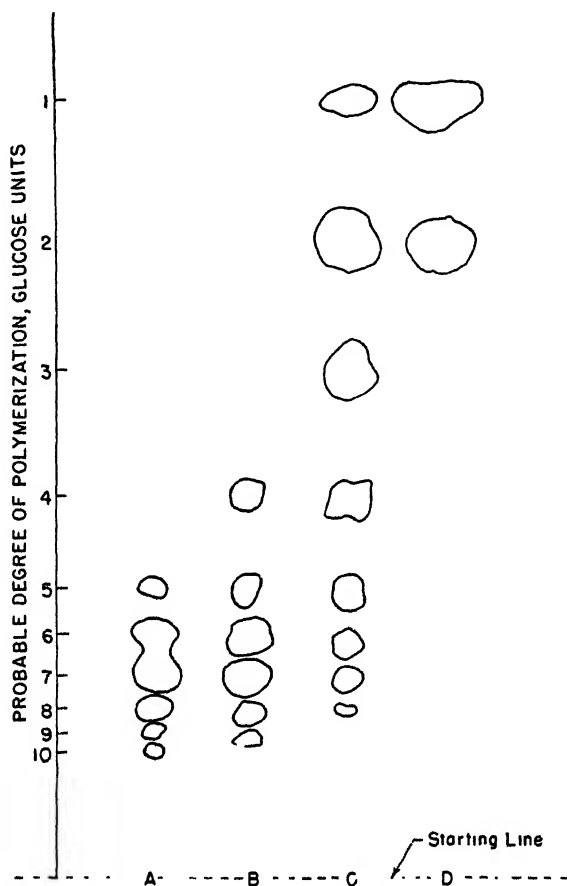


FIG. 1. Paper chromatogram of (A) fraction of amylose hydrolyzate insoluble in both 85% and 95% ethanol, (B) fraction soluble in 85% and insoluble in 95% ethanol, (C) fraction soluble in 95% ethanol, and (D) glucose-maltose mixture. The component sugars are tentatively considered to constitute the homologous series of alpha-1,4-linked glucose polymers as indicated.

showed a reducing power equivalent to 47% conversion to maltose. The yields are only approximate, since they are calculated as percentage of the total weight recovered, which was about 75% of theory. The average degrees of polymerization (D.P.) were calculated from the reducing power, using the reducing power of maltose as a standard. The fraction soluble in 95% ethanol contained, as would be expected,

nearly all of the glucose and maltose formed in the hydrolysis. The fraction insoluble in both 95% and 85% ethanol was essentially free of maltose.

Paper chromatography of these fractions showed clearly that they were all very heterogeneous (Fig. 1). Each contained at least four or five constituents in appreciable amounts, and small quantities of others. It seems most probable that, all together, these compounds constitute the homologous series of glucose, maltose, and alpha-1,4-linked oligosaccharides (5). If that is the case, then it is apparent that the fraction soluble in 95% ethanol contains significant quantities of dextrans containing up to seven glucose units. The least soluble fraction then contains sugars ranging from 4 to 10 glucose units in size. Some chromatograms showed indications of the presence of slower-moving materials which might be still higher saccharides. Studies are now in progress to isolate and characterize more definitely these component sugars and to determine the quantities present.

Paper chromatography thus provides a convenient method for the evaluation of any fractionation method which might be used in studies of the products of the hydrolysis of amylose with alpha-amylase. While similar information apparently can be obtained by the use of interferometric methods in conjunction with carbon column adsorption (16), paper chromatography is simpler in requirements of both equipment and operations.

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## THE EFFECTS OF CHLORINE DIOXIDE ON THE NUTRITIVE VALUE OF WHEAT GLUTEN<sup>1,2</sup>

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### ABSTRACT

The nutritive value of wheat gluten, determined in puppies or in adult dogs, was not altered by treating flour with chlorine dioxide in amounts up to 1.83 gm. chlorine dioxide per hundred weight. There was no evidence of running fits, either clinical, or in electroencephalograms in dogs fed chlorine dioxide-treated wheat gluten. Abnormal electroencephalograms were obtained from dogs fed wheat gluten prepared from flour treated with nitrogen trichloride long before clinical symptoms of abnormalities appeared.

Recent studies have demonstrated that wheat flour treated with nitrogen trichloride, or the gluten therefrom, will produce toxic symptoms when fed to dogs in sufficient quantities. These symptoms are usually described clinically as "running fits" or "canine hysteria" (1-5) and they are associated with an abnormal electroencephalogram (6, 7).

Nitrogen trichloride as a bleaching agent and flour-maturing agent has been superseded by chlorine dioxide. Newell *et al.* (8) have fed dogs, rabbits, rats, and monkeys, flour treated with chlorine dioxide

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<sup>2</sup> Contribution from the Bureau of Biological Research, Rutgers University, New Brunswick, N. J.

for periods up to five and one-half months without producing any abnormal symptoms. Humans fed this treated flour for six weeks were also unaffected. Nakamura and Morris (9) have reported that the feeding of chlorine dioxide-treated flour to either growing or mature dogs did not induce canine hysteria.

The following studies were planned to determine whether or not the treatment of wheat gluten with chlorine dioxide would alter the nutritive quality of the protein or produce changes in the electroencephalograms of dogs upon feeding.

### Materials and Methods

Six twelve week old littermate puppies were fed the diet in Table I *ad lib* (10). The puppies were paired so that one female and one male were fed the same sample of wheat gluten.

TABLE I  
DIET <sup>1</sup>

| Wheat Gluten Diet— <i>ad lib</i> . |          |       | Vitamin Supplements              |                           |
|------------------------------------|----------|-------|----------------------------------|---------------------------|
| Ingredients                        | Calories | Grams |                                  | mg./kg./day               |
|                                    | %        | %     |                                  |                           |
| Wheat gluten                       | 21.93    | 25.0  | Thiamine                         | 0.025                     |
| Dextrose                           | 31.77    | 36.2  | Riboflavin                       | .025                      |
| Dextrin                            | 16.30    | 18.6  | Nicotinic acid                   | .250                      |
| Lard                               | 30.00    | 15.2  | Calcium pantothenate             | .200                      |
| Salt Mixture <sup>2</sup>          |          | 2.0   | Pyridoxine                       | .015                      |
| Agar                               |          | 3.0   | Choline                          | 15.0                      |
|                                    | —        | —     | 2-methyl-1,4-naphthoquinone      | .00001                    |
|                                    | 100.00   | 100.0 | Calcium tocopherol monosuccinate | 0.5                       |
|                                    |          |       | Fish liver oil                   | 470 Units A<br>85 Units D |
|                                    |          |       | Liver extract                    | 0.02 USP Units            |

<sup>1</sup> Mix 1.4 gm. of water with every gram of dog food.

<sup>2</sup> Wesson's Modified Osborne-Mendel Salt Mixture (11).

Nitrogen balance indexes of the gluten were determined on adult dogs according to methods described previously (12). The gluten was water washed from official mill samples of flour containing 65% winter and 35% spring flour. The gluten was prepared in the laboratories of Wallace and Tiernan Company. One sample of flour was untreated, a second was treated with 0.61 gm. of chlorine dioxide per hundred weight, and a third with 1.83 gm. of chlorine dioxide per hundred weight.

Another series of three puppies were fed the wheat gluten from the untreated flour. Electroencephalograms were obtained on these dogs

during a control period and after three and eight weeks feeding of the diet containing this untreated wheat gluten. Another group of three puppies were fed the gluten treated with 1.83 gm. of chlorine dioxide per hundred weight. Electroencephalograms were measured on these as on the control puppies.

Other puppies were placed on a wheat gluten which had been prepared from flour treated with nitrogen trichloride equivalent to 3 gm. per hundred weight. This was done to check the development of hysteria and the effect on the electroencephalogram in dogs.

To determine the encephalograms each dog was anesthetized by intravenous injection of sodium pentothal. While the dogs were asleep electrodes were attached to shaved patches on the right and left frontal areas and the ears, the latter being used as reference points.

After testing the electrode resistance, electroencephalographic records were taken for a period of 20–30 minutes on each dog, at approximately two minute intervals.

### Results and Discussion

The caloric intakes are recorded in Fig. 1. These data illustrate the gradual reduction in the number of calories/day/kg. of body weight as the dogs grew in size. The intake was the same for both sexes and was not altered by treatment with chlorine dioxide. The data in Fig. 2 illustrate the growth of the puppies on these caloric intakes. Treat-

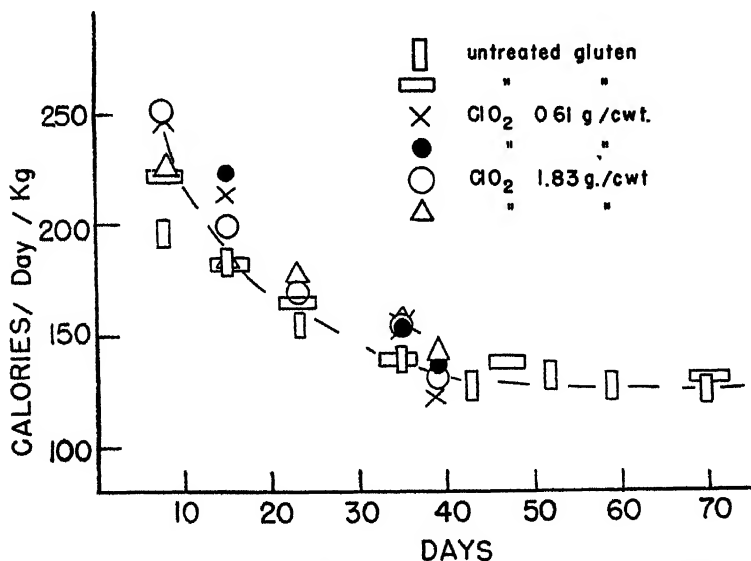


FIG. 1. Decrease in daily caloric intakes of puppies fed diets *ad lib* containing wheat gluten prepared from flour subjected to various treatments



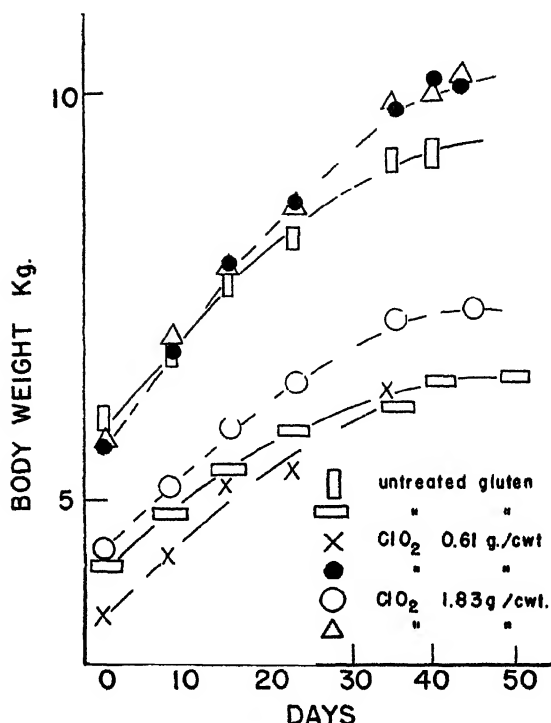


FIG. 2. Growth of puppies fed diet *ad lib* containing wheat gluten prepared from flour subjected to various treatments.

ment of wheat gluten with chlorine dioxide did not effect their growth.

The protein efficiencies recorded in Table II indicate that the nutritive quality of wheat gluten is not altered by treatment with chlorine dioxide. The gain in weight of these puppies on wheat gluten is surprisingly good being approximately the same as on a diet containing whole egg (13). Data presented previously demonstrate, however,

TABLE II

THE EFFECT OF VARIOUS GLUTENS ON NITROGEN INTAKE, WEIGHT GAIN AND PROTEIN EFFICIENCY OF PUPPIES OVER A PERIOD OF 21 DAYS

| Dog No. | Source of Wheat Gluten                        | Nitrogen Intake | Weight Gain | "Protein Efficiency" <sup>1</sup> |
|---------|-----------------------------------------------|-----------------|-------------|-----------------------------------|
|         |                                               | gm.             | gm.         |                                   |
| 85      | Untreated flour                               | 136             | 1,550       | 11                                |
| 86      | Untreated flour                               | 180             | 2,200       | 12                                |
| 87      | Chlorine dioxide-treated flour, 0.61 gm./cwt. | 140             | 1,800       | 13                                |
| 88      | Chlorine dioxide-treated flour, 0.61 gm./cwt. | 211             | 2,800       | 13                                |
| 89      | Chlorine dioxide-treated flour, 1.83 gm./cwt. | 156             | 1,850       | 12                                |
| 90      | Chlorine dioxide-treated flour, 1.83 gm./cwt. | 202             | 2,700       | 13                                |

<sup>1</sup> "Protein efficiencies" are grams body weight gain per gram of nitrogen intake during this period.

TABLE III  
DIGESTIBILITIES AND NITROGEN BALANCE INDEXES<sup>1</sup> OF WHEAT GLUTEN  
PREPARED FROM UNTREATED FLOUR AND FROM FLOUR TREATED  
WITH 1.83 GM. CHLORINE DIOXIDE PER HUNDRED WEIGHT

| Source of Wheat Gluten                        | Digestibility | Absorbed Nitrogen<br>AN | Urinary Nitrogen<br>UN | Protein-free Urinary Nitrogen<br>UN <sub>0</sub> | Nitrogen Balance Index<br>k |
|-----------------------------------------------|---------------|-------------------------|------------------------|--------------------------------------------------|-----------------------------|
| ---                                           | %             | gm./day/<br>sq.M.       | gm./day/<br>sq.M.      | gm./day/<br>sq.M.                                | ---                         |
| Untreated flour                               | 98            | 4.28                    | 4.03                   | 1.88                                             | 0.50 ± .06 <sup>2</sup>     |
| Chlorine dioxide-treated flour, 1.83 gm./cwt. | 98            | 4.24                    | 4.47                   | 2.29                                             | 0.49 ± .05 <sup>2</sup>     |

<sup>1</sup> The values are averages obtained on six adult dogs.

<sup>2</sup> Standard error of mean.

that the growth on whole egg involves a higher protein efficiency, more protein and less fat being laid down with whole egg than with the wheat gluten diets (13).

Digestibilities and nitrogen balance indexes of gluten from untreated and from chlorine dioxide treated flour were determined in adult dogs, the results being recorded in Table III. These data demonstrate further that the digestibility and the retention of nitrogen in the animal is not altered by treating the flour with chlorine dioxide. The indexes of this sample of wheat gluten are slightly higher than the average of 0.44 previously reported for another sample (14).

## B

FIG. 3. Typical electroencephalogram in dogs obtained after three weeks (A) and after eight weeks (B) of feeding chlorine dioxide treated wheat gluten.

Representative samples of electroencephalograms obtained after three and eight weeks of feeding wheat gluten treated with 1.83 gm. chlorine dioxide per hundred weight, are illustrated in Fig. 3. These records are typical of normal dogs. No abnormal records were obtained on dogs fed untreated wheat gluten or wheat gluten prepared from flour treated with chlorine dioxide.

Three animals fed the wheat gluten, treated with nitrogen trichloride developed "running fits" within six to ten days. The dogs were removed immediately from the diet after the abnormal symptoms appeared. Recovery was complete when the animals were placed on the control ration. A typical fit pattern from such a dog is illustrated in Fig. 4. The heightened activity illustrated is common with dogs



FIG. 4. Typical electroencephalogram in dogs obtained after seven days of feeding wheat gluten treated with nitrogen trichloride.

suffering from running fits and is sometimes recorded in these animals several days before clinical symptoms appear. At no time was this increased activity noted in the electroencephalograms from puppies fed the chlorine dioxide-treated wheat gluten and the clinical symptoms of running fits were entirely absent. It can be concluded, therefore, that dogs fed gluten prepared from flour treated with chlorine dioxide at the level employed in these studies (1.83 gm./cwt.) exhibit no abnormalities in cortical activity.

#### Acknowledgment

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# INFLUENCE OF BLEACHING ON THE ABSORPTION SPECTRA OF BUTYL ALCOHOL EXTRACTS OF WHEAT FLOUR<sup>1</sup>

MAX MILNER AND W. W. DODGE

## ABSTRACT

The spectrophotometric characteristics of water-saturated butyl alcohol extracts of wheat flour treated with various concentrations of chlorine dioxide and nitrogen trichloride, showed that the so-called residual pigment which remains in flour after bleaching with high concentrations of these gases is not a carotenoid. This material, probably lipid in nature, has a primary absorption peak in the ultra-violet region somewhat below 260 mμ. In the visible region the effect on this compound of even large dosages of chlorine dioxide, nitrogen trichloride, and benzoyl peroxide was minor. In the ultra-violet spectrum the effect of these agents was marked. Treatment with benzoyl peroxide in amounts used for normal bleaching was most effective in reducing the ultra-violet absorption, whereas very high levels of this oxidizing agent intensified this absorption.

A previous publication (2) dealing with the influence of physical variables on efficiency of flour bleaching with nitrogen trichloride, indicated that massive dosages of this oxidizing gas did not eliminate the light absorption characteristics of water-saturated butyl alcohol extracts of flour samples, when pigment concentration is determined spectrophotometrically by the procedure outlined in Cereal Laboratory Methods (1). Thus, one unbleached flour with a "carotene" concentration of 2.0 p.p.m. when treated with successively larger amounts of this gas showed no color removal beyond 0.65 p.p.m. This color value was retained even with dosages which were approximately 100 times those normally used in commercial bleaching. This heavily over-treated flour had assumed an unnatural pink color, was very rancid, and did not develop a cohesive dough when mixed with water.

The present report deals with a spectrophotometric characterization of the residual pigment and the effect of treatment with nitrogen trichloride, chlorine dioxide, and benzoyl peroxide on the absorption spectrum.

## Materials and Methods

Two unbleached flours commercially milled from average mixes of hard red winter wheat were used in this study. Their composition was as follows:

<sup>1</sup> Manuscript received April 21, 1950. Contribution No. 178, Department of Milling Industry, Kansas Agricultural Experiment Station, Manhattan, Kansas. This research was supported by a grant from the Wallace & Tiernan Co., Inc., Newark, New Jersey.

| Flour<br>sample | Protein <sup>1</sup><br>% | Ash <sup>1</sup><br>% | "Carotene"<br>p.p.m. |
|-----------------|---------------------------|-----------------------|----------------------|
| A               | 10.6                      | 0.40                  | 3.3                  |
| B               | 10.4                      | 0.41                  | 2.7                  |

<sup>1</sup> 14% moisture basis.

Bleaching with nitrogen trichloride was carried out by a regular laboratory procedure previously outlined (2) and flour treatment with chlorine dioxide was performed using the acetic anhydride-sodium chlorite method recently described (4). Bleaching with benzoyl peroxide was accomplished by mixing the flour thoroughly with the required amounts of a commercial preparation consisting of 32% benzoyl peroxide in a mixture containing potassium aluminum sulfate and magnesium carbonate as inert diluents.

Extraction of flour pigments with water-saturated N-butyl alcohol was carried out by the procedure outlined in Cereal Laboratory Methods. The absorption spectra of the extracts measured in terms of optical density were determined with the Beckman Photoelectric Quartz Spectrophotometer, over the wave length interval from 260 to

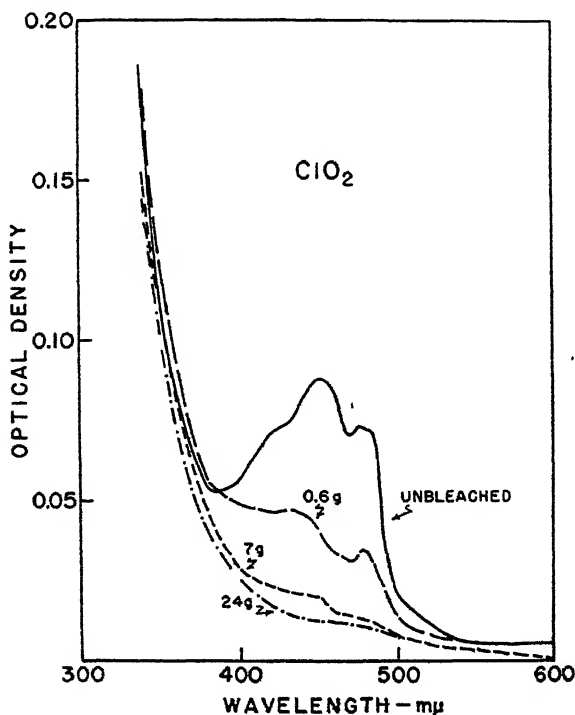


FIG. 1. Influence of various dosages of chlorine dioxide (grams per 100 pounds) on the absorption spectrum of water-saturated butyl alcohol extracts of wheat flour.

600 mu. Readings were taken at intervals not greater than 10 mu throughout the spectral range studied. When operating in the ultra-violet region below 360 mu the mercury vapor radiation source and silica cells were used. For convenience in graphical presentation of the absorption spectra the data obtained in the ultra-violet region below 360 mu are plotted separately from those obtained in the range from 360 to 600 mu.

### Results

The absorption spectra of extracts of unbleached flour "A," and of the same flour bleached with various dosages of chlorine dioxide be-

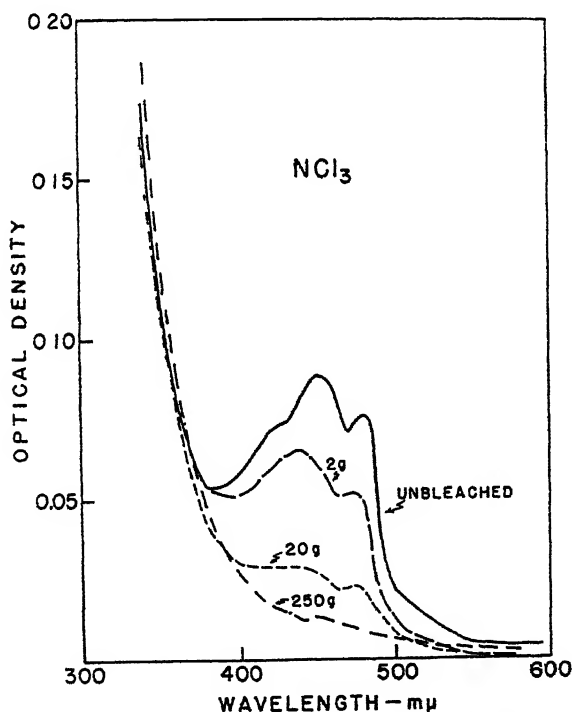


FIG. 2. Influence of various dosages of nitrogen trichloride (grams per 100 pounds) on the absorption spectrum of water-saturated butyl alcohol extracts of wheat flour.

tween 320 and 600 mu, are presented in Fig. 1; similar results obtained with nitrogen trichloride treatment are shown in Fig. 2.

The uppermost curve in both Fig. 1 and Fig. 2 in the range from somewhat below 400 to 600 mu is the typical absorption spectrum of a mixture of xanthophyll pigments, carotene, and other closely related carotenoid materials known to be present in wheat flour. Treatment with increasing concentrations of nitrogen trichloride and chlorine

dioxide, as would be expected, causes a corresponding elimination of this typical spectrum from the flour extracts. It is notable, however, that although the largest dosages of nitrogen trichloride and chlorine dioxide virtually eliminated the spectra due to the carotenoid pigments, the spectrum of another light-absorbing material was revealed, which appeared to be almost unaffected by these oxidizing agents. The absorption intensity of this material increased progressively at wave lengths below 500 m $\mu$ , and was particularly strong as the ultra-violet

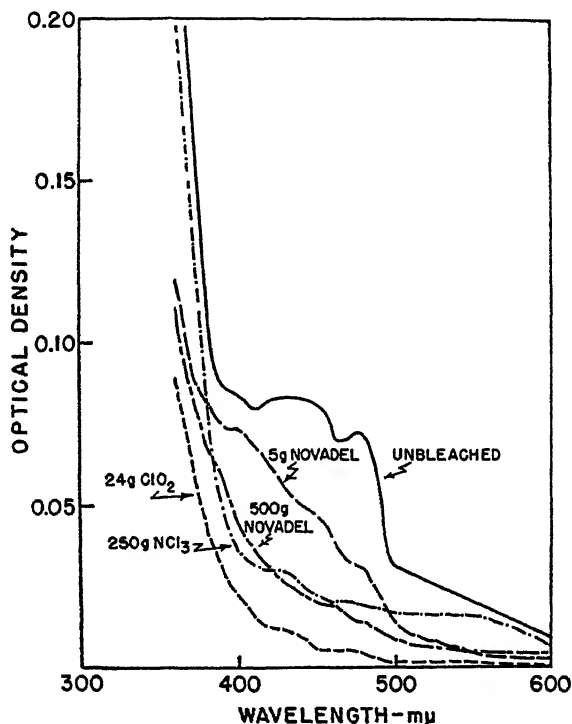


FIG. 3. Influence of various dosages of different bleaching agents (grams per 100 pounds) on the absorption in the visible spectrum of water-saturated butyl alcohol extracts of wheat flour.

region was approached. The spectrum of this compound in the visible range appeared to be unaffected by the very high concentrations of the bleaching gases applied. This absorption was obviously due to the so-called residual pigment in flour which remains after the complete removal of the carotenoids. It exhibited significant light absorption at a wave length of 435.8 m $\mu$  which is that used in the method for pigments prescribed in Cereal Laboratory Methods (1).

The apparently refractory nature of this residual material in the visible range of the spectrum where the carotenoids absorb most

strongly, suggested that an examination in other regions of the spectrum might disclose alterations due to high treatment rates. For this study the "B" flour was used. This flour was bleached with nitrogen trichloride at the rate of 250 gm./cwt. and with chlorine dioxide at the rate of 24 gm./cwt. These treatments were approximately 100 times the normal commercial levels. In addition, a sample was treated with benzoyl peroxide mixture at the normal rate of 5 gm./cwt. as well as at 500 gm./cwt. The absorption spectra in the visible range between

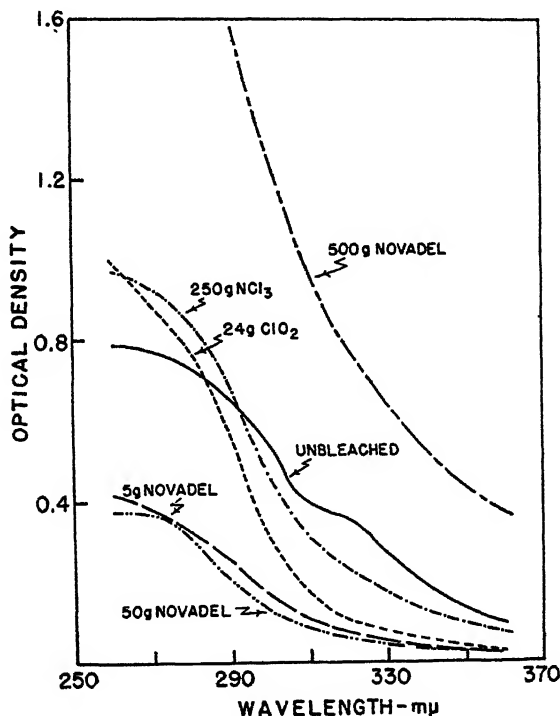


FIG. 4. Influence of various dosages of different bleaching agents (grams per 100 pounds) on the absorption in the ultra-violet spectrum of water-saturated butyl alcohol extracts of wheat flour.

360 and 600 mμ of the extracts of the treated and untreated flours prepared several days after treatment are shown in Fig. 3, and the spectra in the ultra-violet region from 250 to 360 mμ are shown in Fig. 4. The data in Fig. 4 were obtained by diluting the original extracts in the ratio of 1:4. The absorption characteristics at wave lengths shorter than 260 mμ could not be determined due to the strong absorption below this wave length by the solvent itself.

The data of Figs. 3 and 4 indicate that benzoyl peroxide in normal concentrations apparently possesses an efficiency in oxidizing the caro-



tenoid pigments comparable to that of nitrogen trichloride and chlorine dioxide. At the 100-fold normal level of treatment the spectra of the extracts from the benzoyl peroxide and nitrogen trichloride-treated samples are similar as regards shape and intensity when the wave length region characteristic of the carotenoid pigments is considered. However, similar treatment with chlorine dioxide appeared to cause a definite alteration in the absorption band of the residual pigment. In this case the absorption curve was similar in shape but was significantly reduced in intensity.

In comparison to the minor effect of the residual pigment on the absorption spectrum at wave lengths above 360  $\mu$ , the changes below 360  $\mu$  were marked (Fig. 4). Treatment with high levels of nitrogen trichloride and chlorine dioxide caused a narrowing and elevation of the absorption peak in comparison to that due to untreated flour. Benzoyl peroxide mixture at dosages of 5 and 50 gm./cwt. caused a drastic and nearly equal reduction in absorption intensity, whereas the 500 gm. treatment with benzoyl peroxide mixture resulted in a very marked intensification of the absorption band. These results indicate that normal dosages of benzoyl peroxide are more efficient than are nitrogen trichloride and chlorine dioxide in the destruction of the ultra-violet light-absorbing pigment, but that extremely massive dosages of this chemical apparently cause formation of a derivative which has much stronger light absorption characteristics.

### Discussion

The present study indicates that the so-called residual pigment whose absorption is virtually unaltered at 435.8  $\mu$  when bleached with nitrogen trichloride and chlorine dioxide, has a primary absorption band in the ultra-violet region centered somewhat below 260  $\mu$ . This absorption peak was noted a number of years ago in ethyl alcohol extracts of wheat (3) and probably is due to sterols or other materials related to fats.

It is probable that two effects are responsible for the changes in the absorption spectra observed in the region from 360 to 260  $\mu$ . Certain modes of oxidation may produce substances from carotenoid pigments exhibiting low absorption in the region where carotenoid absorption is normally high (440 to 500  $\mu$ ) and high absorption in the ultra-violet. Similarly, the nature of the oxidant employed may differentially affect the lipid fraction in the two spectral regions. It is probable that nitrogen trichloride and chlorine dioxide which had no detectable influence on the absorption spectrum of the lipid fraction (residual pigment) in the carotenoid absorption range, would also have little effect on this fraction in the ultra-violet region, and that the

changes in spectral characteristics in the latter range are due to carotenoid oxidation products. Similarly benzoyl peroxide in the lower range of concentration (5 gm. and 50 gm.) probably yields carotenoid oxidation products of reduced absorbing power in the ultra-violet region. On the other hand, very high dosage of benzoyl peroxide (500 gm.) probably causes profound oxidative changes in the lipid fraction, yielding the very high absorption noted in this region.<sup>2</sup>

The present studies suggest that the efficiency of benzoyl peroxide as a flour bleaching agent is about the same as that of the gaseous agents, nitrogen trichloride and chlorine dioxide, when the removal of the carotenoid pigments only is considered. It is of interest, nevertheless, that this reagent is considerably more efficient than are the gaseous materials in reducing the radiation-absorbing intensity of materials which absorb primarily in the ultra-violet region.

These results suggest that further studies are needed to clarify the differences in the effects of nitrogen trichloride, chlorine dioxide, and benzoyl peroxide on the isolated pigments and other radiation-absorbing compounds of wheat.

#### Acknowledgments

The authors gratefully acknowledge the generosity of Mr. John S. Whinery of the Rodney Milling Company and Mr. John Creech of the Standard Milling Company in supplying the commercial flours used in this study, and the cooperation of Dr. H. L. Mitchell in making a Beckman spectrophotometer available to them.

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<sup>2</sup> The authors are indebted to G. N. Irvine of the Grain Research Laboratory, Winnipeg, for suggesting the interpretation outlined in this paragraph.

## STUDIES ON A RAPID TEST FOR THE VIABILITY OF CORN FOR INDUSTRIAL USE <sup>1,2</sup>

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### ABSTRACT

The 2,3,5-triphenyltetrazolium chloride test for viability is suggested as a means of detecting corn kernels that have been killed by high temperature during drying or by freezing, and for determining the percentage of dead kernels in corn before purchase. The test with the reagent is simple and requires only four hours for completion.

Corn processors have long needed a rapid test to determine, before purchase, the suitability of grain for their use. In the past few years this need has grown as the result of the increasingly prevalent practice of drying corn artificially. Several factors have contributed to make artificial drying a more common practice, chief of which is the present widespread use of mechanical corn pickers. Harvest with a corn picker is completed in about one-third, or less, the length of time formerly required for hand picking. Hence much of the corn has less time to dry in the field.

The number of publications on the structure and use of corn driers is witness to the prevalent need for drying corn before it is stored. No problem would exist if recommendations (3, 4, 10) were heeded, but a tendency to use too high temperatures, both on the farm and at elevators, persists.

Some corn wet millers have been loathe to purchase corn from a county in which a commercial drier was known to be operating because over-heating during drying damages corn for starch production. Processors have stated that dead corn is undesirable for the production of starch. These facts, together with the experience of seed corn producers that over-heating destroys viability, led to the investigation of a test for viability of corn which might serve to indicate the value of the corn for industrial processing. The present data are presented as a progress report.

### Materials and Methods

*Viability Test.* The viability test with 2,3,5-triphenyltetrazolium chloride, which was developed and applied to corn by Lakon (5, 6, 7), was selected because the reagent gives an easily discerned carmine red

<sup>1</sup> Manuscript received May 19, 1950.

<sup>2</sup> Contribution from the Northern Regional Research Laboratory, Peoria, Illinois. One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

color to living portions of the germ. Lakon and others have described the test as requiring a total of 7 to 18 hours for completion. Our preliminary experiments had shown that the total time required could be reduced to a maximum of about 4 hours. One hundred kernels of the corn to be tested were soaked for from 1 to 2 hours in distilled water. The shorter period was used when soft corn<sup>1</sup> was tested, the longer when naturally or artificially-dried corn was being examined. After soaking, each kernel was bisected longitudinally perpendicular to the broad face. The cut was made with a sharp razor blade while the kernel was held under distilled water. Bisecting under distilled water tended to prevent the formation of a deposit over the cut surface.

One half of each kernel was laid, cut side down, in a petri dish containing sufficient 1% aqueous triphenyltetrazolium chloride solution to cover the kernel pieces. The dish was then placed in a dark cabinet at room temperature for from 1 to 1½ hours. The kernel pieces were then examined to determine viability. The variation between duplicate samples was within the range, 0-5%.

The viable parts are colored carmine red by this procedure. In strongly viable corn, (i.e., kernels which will give vigorous seedlings), the entire germ, composed of the embryonic plant and the scutellum, is colored. There are certain instances, however, when only portions of the germ are colored yet the seed is still viable. Typical staining patterns of kernels bordering between viability and non-viability are shown in Fig. 1. It is these patterns which are most difficult to interpret and which, therefore, require the deepest coloration with the reagent. In such cases, the red color appears (1) in all parts of the scutellum, and in all of the embryonic axis except the radicle (the embryonic root); (2) in only the medial zone of the scutellum, and in all of the embryonic axis (Fig. 1-2); and (3) in only the medial zone of the scutellum, and in all but the radicle of the embryonic axis (Fig. 1-3). If only strongly viable corn is considered to be suitable for use, the period of contact of the kernel pieces with the reagent can be limited to 30 minutes. In this case only those pieces which show a pink color over the entire cut surface of the germ would be counted as viable.

In cases of urgency at least a close estimate of the viability of corn can be made with triphenyltetrazolium chloride in only one hour. The corn is bisected without the preliminary soaking, and the viability is determined after a one-hour staining period. There is some mechanical difficulty in bisecting the hard kernel. The resulting damage to the cells sometimes causes formation of a heavy scum which must be removed before the viability of the kernel is determined.

<sup>1</sup> "The term soft corn can be applied properly to any corn that is soft and watery, in contrast to the relatively hard dry kernels of ears as they are cribbed under ordinary conditions" (9).

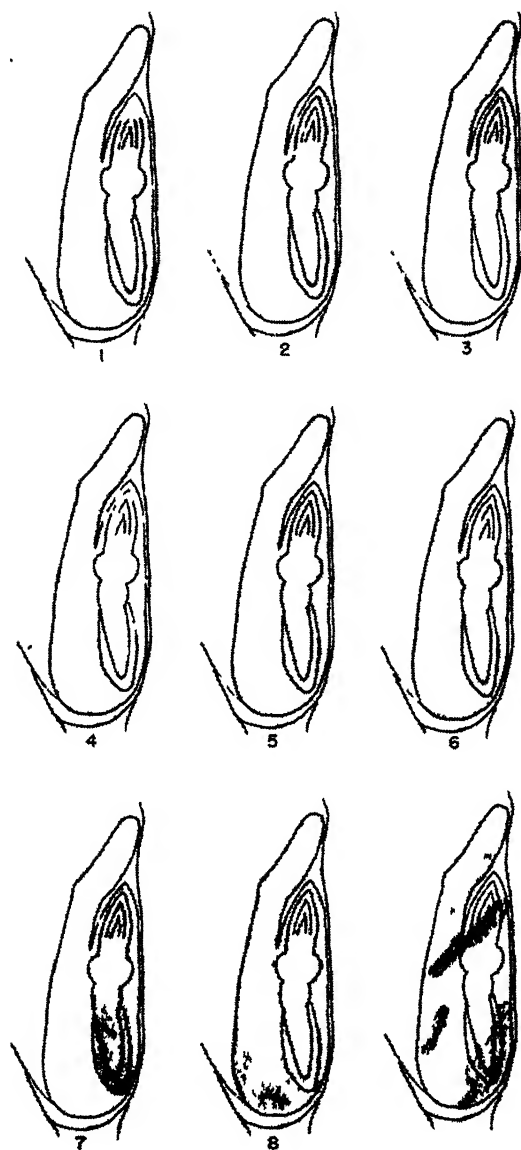


FIG. 1. Typical coloration of corn germ in border-line cases. Shaded portion represents the area colored carmine red by the test.

1. Viable—surface tissues which do not readily take up the reagent exposed by improper bisection.
2. Viable—medial portion of scutellum and embryonic axis colored.
3. Viable—all colored except radicle and lower scutellum.
4. Non-viable—no part of embryonic axis colored.
5. Non-viable—a lateral bud uncolored.
6. Non-viable—plumule uncolored.
7. Non-viable—entire scutellum uncolored.
8. Non-viable—mesocotyl region uncolored.
9. Non-viable—streaked, with no essential part completely colored.

Care must be taken in bisecting the kernels. If the upper portion of the embryonic plant is not cut through, surface tissues may be exposed which do not readily take up the reagent (Fig. 1-1).

In testing corn heavily contaminated with bacteria, a colored deposit may form over the cut surface. This deposit need not interfere with the test; it can be scraped away before the kernel is examined (7).

The reagent solution is sensitive to light, but it can be kept for several months in a dark bottle stored in a refrigerator. It is colorless when prepared; appearance of a dark amber color indicates that it is no longer useful (7).

Corn which has previously been frozen often gives a purplish-red color, rather than the usual carmine red, when treated with the reagent.

Standard germination tests were run on each sample as a standard in judging the accuracy of the triphenyltetrazolium chloride test. One hundred kernels were placed between moist layers of cheesecloth, or similar material, and the percent of kernels germinated was determined at the end of 5 to 7 days. This standard germination test usually agreed with the color test for viability within 10%.

*Corn Tested.* Four samples of naturally soft, shelled corn, not moldy, containing from 18.6 to 30.3% moisture, were dried immediately upon receipt at several temperatures, at 10° intervals from 110° to 220°F., to approximately 13% moisture content, each in 1 × 5 × 5-inch containers made of quarter-inch wire cloth, which were filled and placed in a forced-draft oven maintained at the required temperature. One sample was also obtained after commercial drying, and one which had been dried artificially on a farm.

Attempts were made to increase the number of samples by tempering naturally-dried corn to a higher moisture content. These were abandoned when it was found that soft corn and tempered corn of the same moisture content behaved dissimilarly as to viability even during short periods of storage.

*Processing.* The laboratory wet-milling process method used was that described by Cox, MacMasters, and Hilbert (2). In most cases, the steeping solution contained 0.15% sulfur dioxide; more rarely a 0.10% sulfur dioxide solution was used.

## Results and Discussion

Under the conditions of drying used, temperatures above approximately 120° to 140°F., depending upon the individual sample, greatly decreased the viability of the corn. Typical data are shown graphically in Fig. 2. A correlation between viability and ease of processing of corn is strongly suggested, since Shedd (10) and Hukill (4) state that corn dried at temperatures above 130°F. is damaged for at least certain types of processing.

It is known, although too often overlooked in drying practice, that the effect of drying temperature upon viability is influenced by the moisture content of the grain (3, 8). This may explain some results which are otherwise anomalous from the standpoint of both viability and ease of processing.

It is impossible to draw final conclusions from the small number of samples which were processed. Certain observations made during the laboratory processing studies suggest possible effects of drying temperature upon the processing behavior of corn. Corn dried at temperatures above approximately 130°F. usually gave harder grits and more

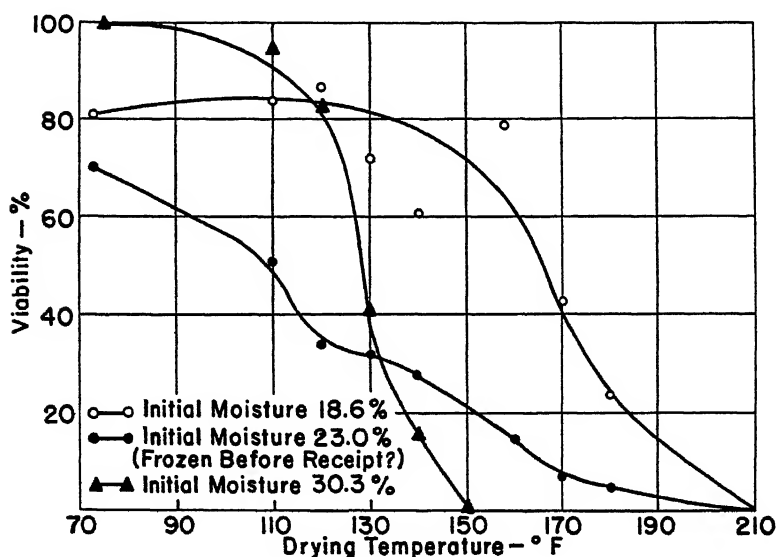


FIG. 2. Effect of drying temperature on viability of corn.

rubbery germ than that dried at lower temperatures. While no germ flotation was attempted, it was noted that the rubbery germs tended to sink when suspended in the ground corn slurry. There was also a marked tendency for oil to be found in starch separated from the over-heated corn. The reported difficulty in the industrial processing of artificially-dried corn may well be attributable, therefore, to the fact that but little attention has been given in the past to control of the temperature when drying corn destined for use other than as seed.

Freezing, as well as over-heating, may decrease the viability of corn. In the present experiments, it was found that corn containing more than about 23% moisture after being frozen, decreased rapidly in viability. At lower moisture contents of the corn, the decrease in viability after

freezing was relatively slow. Development of a purplish-red color during the triphenyltetrazolium chloride test has been observed only in corn which has previously been frozen (cf. 1). Some frozen corn, however, gives a carmine red color with the reagent, hence the test is not specific for the detection of corn damaged by freezing.

There are no figures available on the actual economic disadvantage of processing dead corn. However, industrial experience leads to the definite conclusion that dead corn causes departures from normal milling results. Corn dried at temperatures too high gives poor quality and low yields of starch when wet milled. Here, too, the germ has been killed and the determination of viability may give an indication of milling quality. A means of determining the condition of commercial corn is therefore of direct economic importance.

On the basis of the knowledge at hand, the test for viability with 2,3,5-triphenyltetrazolium chloride solution appears to offer promise for the determination of the industrial suitability of corn as offered for sale. This test requires no complicated equipment, relatively little time, and a minimum of technical knowledge on the part of the operator.

#### Acknowledgments

The authors gratefully acknowledge the laboratory assistance of Margaret Holzappel and the help of the Analytical and Physical Chemical Division of this Laboratory in procuring samples and making chemical analyses of the corn.

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## COMMUNICATION TO THE EDITOR

### Stabilization of Solutions of Wheat Gluten in Dilute Acetic Acid by Brief Heat Treatment

SIR:

Rose and Cook (5) first reported that solutions of wheat gluten in dilute acetic acid are unstable. Evidence for the instability was a drop in viscosity over a period of time. Hydrolytic changes were also measurable (1). Olcott, Sapirstein, and Blish (4) concluded that the changes were catalyzed by a heat-sensitive enzyme, since the solutions were almost completely stabilized by heat treatment (5–10 minutes at 100°). This observation suggested that acetic acid solutions of wheat gluten to be used in research investigations should be heat-treated as soon as possible after their preparation to destroy the enzyme.

A simple set-up for accomplishing this end has been used routinely in this laboratory for the past 8 years (Fig. 1). The rate of flow of the gluten solution is regulated either by means of the clamp or by adjusting the height of the reservoir. In two sets of early experiments it was shown that the gluten solution need be heated for only a very brief period to achieve stabilization. Data are shown in Table I. With the most rapid flow the gluten solution was in the steam chamber only 11 seconds. However, it was not possible, even with the more severe treatments, to stabilize the solutions completely. The cause of the

TABLE I

|                      | Rate of Flow | Time in Steam Chamber | Kinematic Viscosity after Elapsed Time in Days at 25° |      |      |      | Total Decrease |
|----------------------|--------------|-----------------------|-------------------------------------------------------|------|------|------|----------------|
|                      |              |                       | 0                                                     | 1    | 3    | 5    |                |
| Exp. I <sup>1</sup>  | ml./min.     | min.                  |                                                       |      |      |      |                |
|                      | Control      | —                     | 2.48                                                  | 1.98 | 1.66 | 1.55 | 0.93           |
|                      | 31           | 7                     | 2.31                                                  | 2.29 | 2.26 | 2.22 | .09            |
|                      | 90           | 2.3                   | 2.46                                                  | 2.41 | 2.35 | 2.32 | .14            |
| Exp. II <sup>2</sup> | 220          | 1                     | 2.50                                                  | 2.46 | 2.41 | 2.38 | .12            |
|                      | Control      | —                     | 3.91                                                  | 3.19 | —    | 2.44 | 1.47           |
|                      | 17           | 2.3                   | 3.92                                                  | 3.72 | 3.62 | 3.59 | .33            |
|                      | 44           | 0.91                  | 4.08                                                  | 3.85 | 3.71 | 3.66 | .42            |
|                      | 90           | 0.44                  | 4.18                                                  | 3.93 | 3.78 | 3.74 | .44            |
|                      | 210          | 0.19                  | 4.19                                                  | 3.91 | 3.76 | 3.68 | 0.51           |

<sup>1</sup> The original solution contained 4.2% gluten ( $N \times 5.7$ ) in 0.1 *N* acetic acid. After heat treatment, the various samples were diluted with an equal volume of 0.1 *N* acetic acid for the viscosity determinations. Volume of the steam chamber, 210 ml.

<sup>2</sup> Gluten was dissolved in 0.05 *N* acetic acid solution, to make approximately 3.5% solution (pH 4.0). Volume of steam chamber, 40 ml.

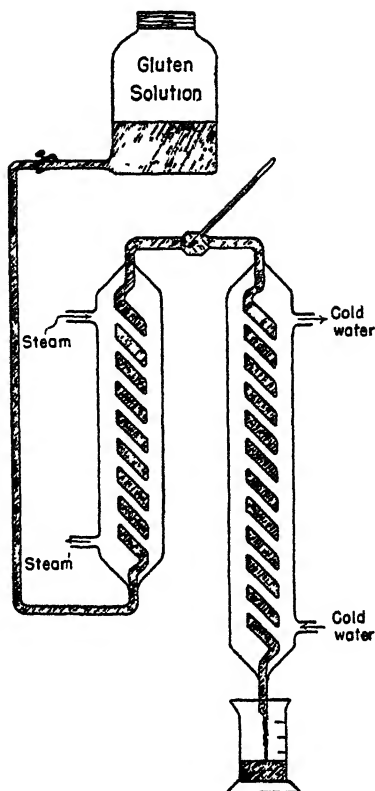


FIG. 1. Apparatus used for continuous rapid heat treatment of gluten solutions.

slow but significant drop in viscosity of heated solutions is not known. Microbiological contamination was suspected but it was found that no organisms could be detected in stained smears of the solution and also that the presence of Merthiolate as a preservative did not change the rate of fall of viscosity.

Lusena (2) and Lusena and Adams (3) have recently published methods for obtaining dry gluten preparations by freeze- or spray-drying acetic acid dispersions of the gluten. The adoption of the heat-treatment step in their procedures would result in more uniform products, insofar as the magnitude of degradative changes during subsequent handling would be diminished.

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August 14, 1950

## BOOK REVIEWS

**A German-English Dictionary for Chemists**, 3rd edition, by Austin M. Patterson. 541 pp. John Wiley and Sons, Inc., New York, Chapman and Hall, Ltd., London. Price \$5.00.

This book is designed chiefly for chemists and chemical engineers but it is also useful for workers in other fields of science such as physics, biology and geology. Compared with the second edition which had 42,000 entries, this third edition has 59,000. The increase has resulted partly from additional meanings to words listed in the former edition, but chiefly from new additions in the fields of chemical technology, electronics and warfare. Still carrying a good vocabulary of general words, this dictionary, which is well-printed, serves as a great stand-by for all except the experts in the language who are interested in German scientific literature.

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August 3, 1950

**"A Textbook of Biochemistry."** By Philip H. Mitchell. 2nd edition, 1950. McGraw-Hill Book Co., Inc. \$6.00. xvii + 695 pp.

The second edition of this generally well-accepted book retains the arrangement and sequence of subject matter used in the first edition published in 1946. The 21 chapters of the book deal with carbohydrates, photosynthesis, fats and related substances, proteins and amino acids, nucleoproteins and nucleic acids, the vitamins, enzymes, digestion, absorption and detoxication, blood and lymph, chemistry of respiration and acid-base balance, biological oxidation, calorimetry and energy metabolism, carbohydrate metabolism, metabolism of lipids, protein metabolism, urine, composition of foods; dietetics, the chemistry of animal tissues, chemistry of the hormones, and chemotherapy. As might be gathered from the table of contents, the subject of biochemistry in this book is treated almost exclusively from the standpoint of animal biochemistry and the chapter on photosynthesis appears to be almost superfluous in this context. The second edition covers 55 pages more than the first but this enlargement is accounted for in part by the greater marginal spaces on each page and the expansion in text is partly compensated by a regrettable abbreviation of the subject index.

According to the author "Emphasis in this text has been given and major space allotted to such subjects as the constitution and activities of enzymes, the intermediary reactions of anabolism and catabolism and the vital significance of hormones and vitamins." In spite of this avowed purpose of the book, most of the material in the second edition has not been greatly changed to reflect the tremendous research activity and progress which have taken place in the four year interval between the first and second edition. Thus, the highly significant study of localization of enzymatic and other cellular constituents in such particles as mitochondria, microsomes and nuclei appears to be overlooked. The paragraph on page 660 on the outlook in chemotherapy remains essentially unchanged from the first edition in spite of the great advances which have been made in this field. The treatment of the present concept of high energy phosphate bonds is extremely sketchy and the reactions concerned in their regeneration are almost completely neglected. The reaction between phosphopyruvic acid and ADP (p. 441) is still pictured as being irreversible. In the second edition many errors occurring in the first edition have been eliminated while others unfortunately appear unchanged. Thus, "Dimedon" is erroneously referred to on page 55 as "dimethylhydroxyresorcinol"; the Reichert Meissl number is described as a measure of the "volatile fatty acid content of a fat" and decanoic acid is included among the acids contributing to it (p. 77). On page 365 the carbohydrate component of coenzyme I is written with the formula of D-lyxose but is labeled as D-ribose.

A very admirable feature of Dr. Mitchell's book is the numerous references to review articles and to research papers given at the end of each chapter, but here, too, the reviewer feels that many of the older references could have profitably been replaced by those which would guide the student to the more recent work. In spite of the shortcomings which have been pointed out, Dr. Mitchell's book is particularly suitable for use by nonmedical students of biochemistry. The reviewer, for instance, will continue to recommend its use for supplementary reading to a class of undergraduate students taking a course in "animal biochemistry."

The physical makeup of the book and the binding are good and few typographical errors have been noted.

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September 28, 1950

## ERRATA

Cereal Chemistry, Vol. 27, No. 5

(September, 1950)

Page 370. In revising their manuscript, Irvine *et al.* made certain changes in Table II, page 371, but unintentionally overlooked alterations in the text in which these data are discussed. Paragraph two should read:

"Table II lists data for the mixing reaction. The rate of the mixing reaction is largely independent of pigment level and varies, less widely than the rate of the initial reaction for the samples studied, from 0.300 to 0.501 p.p.m. per minute, a factor of slightly less than two. Cyanide inhibits the rate of mixing reaction for four of the six varieties as much as 53%; overall inhibition of the reaction to ten minutes mixing by cyanide is marked for all of the varieties tested, and varies from 27 to 52%. Inhibition of the mixing reaction by 30% alcohol is very marked in all cases, ranging from 70 to 100%, and the net balance between acceleration of the initial reaction and inhibition of the mixing reaction after ten minutes of mixing shows an over-all inhibition in all cases, varying from 17 to 41%.

Please make the following corrections in the paper by Fifield *et al.*:

Page 388. Line 11, for "17" read "77."

Page 389. Line 9, for "load" read "loaf."

## Cereal Chemistry

### EDITORIAL POLICY

*Cereal Chemistry* publishes scientific papers dealing with raw materials, processes, or products of the cereal industries, or with analytical procedures, technological tests, or fundamental research, related thereto. Papers must be based on original investigations, not previously described elsewhere, which make a definite contribution to existing knowledge.

*Cereal Chemistry* gives preference to suitable papers presented at the Annual Meeting of the American Association of Cereal Chemists, or submitted directly by members of the Association. When space permits, papers are accepted from other scientists throughout the world.

The papers must be written in English and must be clear, concise, and styled for *Cereal Chemistry*.

Manuscripts for publication should be sent to the Editor in Chief. Advertising rates may be secured from and subscriptions placed with the Managing Editor, University Farm, St. Paul 1, Minnesota. Subscription rates, \$9.00 per year. Foreign postage, 50 cents extra. Single copies, \$2.00; foreign, \$2.10.

### SUGGESTIONS TO AUTHORS

**General.** From January 1, 1948, an abstract will be printed at the beginning of each paper instead of a summary at the end, references will be numbered to provide the option of citing by number only, and date of receipt, author's connections, etc., will be shown in footnotes. Except on these points, authors will find the last volume of *Cereal Chemistry* a useful guide to acceptable arrangements and styling of papers. "On Writing Scientific Papers for Cereal Chemistry" (*Trans. Am. Assoc. Cereal Chem.* 6: 1-22, 1948) amplifies the following notes.

Authors should submit two copies of the manuscript, typed double spaced with wide margins on 8½ by 11 inch white paper, and all original drawings or photographs for figures. If possible, one set of photographs of figures should also be submitted. Originals can then be held to prevent damage, and the photographs can be sent to reviewers.

**Titles and Footnotes.** Titles should be specific, but should be kept short by deleting unnecessary words. The title footnote shows "Manuscript received . . ." and the name and address of the author's institution. Author footnotes, showing position and connections, are desirable although not obligatory.

**Abstract.** A concise abstract of about 200 words follows title and authors. It should state the principal results and conclusions, and should contain, largely by inference, adequate information on the scope and design of the investigation.

**Literature.** In general, only recent papers need be listed, and these can often be cited more advantageously throughout the text than in the introduction. Long introductory reviews should be avoided, especially when a recent review in another paper or in a monograph can be cited instead.

References are arranged and numbered in alphabetical order of authors' names and show author, title, journal, volume, first and last pages, and year. The list is given at the end of the paper. Reference numbers must invariably be cited in the text, but authors' names and year may be cited also. Abbreviations for the names of journals follow the list given in *Chemical Abstracts* 40: I-CCIX. 1946.

**Organization.** The standard organization involves main sections for abstract, introduction, materials, methods, results, discussion, acknowledgments, and literature cited. Alternately, a group of related studies, each made with different materials or methods, may require a separate section for each study, with subsections for materials and methods, and for results, under each section. Center headings are used for main sections and italicized run-in headings for subsections, and headings should be restricted to these two types only.

**Tables.** Data should be arranged to facilitate the comparisons readers must make. Tables should be kept small by breaking up large ones if this is feasible. Only about eight columns of tabular matter can be printed across the page. Authors should omit all unessential data such as laboratory numbers, columns of data that show no significant variation, and any data not discussed in the text. A text reference can frequently be substituted for columns containing only a few data. The number of significant figures should be minimized. Box and side heading should be kept short by abbreviating freely; unorthodox abbreviations may be explained in footnotes, but unnecessary footnotes should be avoided. Leader tables without a number, main heading, or ruled lines are often useful for small groups of data.

Tables should be typed on separate pages at the end of the manuscript, and their position should be indicated to the printer by typing "(TABLE I)" in the appropriate place between lines of the text. (Figures are treated in the same way.)

**Figures.** If possible, all line drawings should be made by a competent draftsman. Traditional layouts should be followed: the horizontal axis should be used for the independent variable; curves should be drawn heaviest, axes or frame intermediate, and the grid lines lightest; and experimental points should be shown. Labels are preferable to legends. Authors should avoid identification in cut-lines to be printed below the figure, especially if symbols are used that cannot readily be set in type.

All drawings should be made about two to three times eventual reduced size with India ink on white paper, tracing linen, or blue-lined graph paper; with any other color, the unsightly mass of small grid lines is reproduced in the cut. Lettering should be done with a guide using India ink; and letters should be  $\frac{1}{8}$  to  $\frac{1}{16}$  inch high after reduction.

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**Text.** Clarity and conciseness are the prime essentials of a good scientific style. Proper grouping of related information and thoughts within paragraphs, selection of logical sequences for paragraphs and for sentences within paragraphs, and a skillful use of headings and topic sentences are the greatest aids to clarity. Clear phrasing is simplified by writing short sentences, using direct statements and active verbs, and preferring the concrete to the abstract, the specific to the general, and the definite to the vague. Trite circumlocutions and useless modifiers are the main causes of verbosity; they should be removed by repeated editing of drafts.

**Editorial Style.** A.A.C.C. publications are edited in accordance with *A Manual of Style*, University of Chicago Press, and *Webster's Dictionary*. A few points which authors often treat wrongly are listed below:

Use names, not formulas, for text references to chemical compounds. Use plural verbs with quantities (6.9 g. were). Figures are used before unit abbreviations (3 ml.), and % rather than "per cent" is used following figures. All units are abbreviated and followed by periods, except units of time, which are spelled out. Repeat the degree sign ( $5^{\circ}$ - $10^{\circ}$  C.). Place 0 before the decimal point for correlation coefficients ( $r = 0.95$ ). Use \* to mark statistics that exceed the 5% level and \*\* for those that exceed the 1% level; footnotes explaining this convention are no longer required. Type fractions on one line if possible, e.g.,  $A/(B + C)$ . Use lower case for farinograph, mixogram, etc., unless used with a proper name, i.e., Brabender Farinograph. When in doubt about a point that occurs frequently, consult the Style Manual or the Dictionary.

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